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(57) Abstract

Restin, a novel anti–angiogenic protein is described, as well as fragments thereof. Methods for expression of the proteins at high titer are also described.

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RESTIN AND METHODS OF USE THEREOF

RELATED APPLICATIONS

This application claims priority to application 60/067,888, filed December 8, 1997, 60/082,663, filed April 22, 1998, and 60/108,536, filed November 16, 1998, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The prognosis for metastatic cancer remains highly unfavorable. Despite advances in radiation therapy and chemotherapy, the long term survival of treated patients has shown only marginal improvement over the past few decades. The lack of significant treatment options available for metastatic cancers emphasizes the need to focus on the development of novel therapeutic strategies. In this regard, targeting tumor vasculature of solid tumors has recently shown promising results in several animal model systems (Baillie *et al.* (1995) *Br. J. Cancer* 72:257-67; Bicknell, R. (1994) *Ann. Oncol.* 5 (Suppl.) 4:45-50; Fan *et al.* (1995) *Trends Pharmacol. Sci.* 16:57-66; Thorpe, P.E. and Burrows, F.J. (1995) *Breast Cancer Res. Treat.* 36:237-51; Burrows, F.J. and Thorpe, P.E. (1994) *Pharmacol. Ther.* 64:155-74). In a nude mouse model, for instance, introduction of a wild type VHL gene into 786-0 cells, a RCC tumor cell line, inhibited tumor growth (Iliopoulos *et al.* (1995) *Nat. Med.* 1:822-26) and angiogenesis.

The growth of solid tumors beyond a few mm^3 depends on the formation of new blood vessels (Folkman, J. (1971) *N. Engl. J. Med.* 285:1182-86). Numerous studies have shown that both primary tumor and metastatic growth are angiogenesis-dependent (Folkman, J. (1971) *N. Engl. J. Med.* 285:1182-86; Folkman, J. (1972) *Ann. Surg.* 175:409-16; Folkman, J. and Shing, Y. (1992) *J. Biol. Chem.* 267:10931-34; Folkman, J. (1996) *Sci. Am.* 275:150-54). A number of angiogenesis inhibitors have been identified. Certain ones, such as platelet factor-4

(Maione *et al.* (1990) *Science* 247:77-79; Gupta *et al.* (1995) *Proc. Natl. Acad. Sci. (USA)* 92:7799-7803), interferon α, interferon-inducible protein-10, and PEX (Angiolillo *et al.* (1995) *J. Exp. Med.* 182:155-62; Strieter *et al.* (1995) *Biochem. Biophys. Res. Commun.* 210:51-57; Brooks *et al.* (1998) *Cell* 92:391-400), are not "associated with tumors," whereas two others, angiostatin and endostatin, are "tumor-associated" (O'Reilly *et al.* (1994) *Cell* 79:315-28; O'Reilly *et al.* (1997) *Cell* 88:277-85). Angiostatin, a potent endogenous inhibitor of angiogenesis generated by tumor-infiltrating macrophages that upregulate matrix metalloelastase (Dong *et al.* (1997) *Cell* 88:801-10), inhibits the growth of a wide variety of primary and metastatic tumors (Lannutti *et al.* (1997) *Cancer Res.* 57:5277-80; O'Reilly *et al.* (1994) *Cold Spring Harb. Symp. Quant. Biol.* 59:471-82; O'Reilly, M.S., (1997) *Exs.* 79:273-94; Sim *et al.* (1997) *Cancer Res.* 57:1329-34; Wu *et al.* (1997) *Biochem. Biophys. Res. Commun.* 236:651-54).

Recently, O'Reilly, *et al.* ((1997) *Cell* 88:277-85) isolated endostatin, an angiogenesis inhibitor from a murine hemangioendothelioma cell line (EOMA). Circulating levels of a fragment of human endostatin have been detected in patients with chronic renal insufficiency with no detectable tumor, but this fragment had deletions, and no anti-angiogenic activity (Standker *et al.* (1997) *FEBS Lett.* 420:129-33). The amino terminal sequence of endostatin corresponds to the carboxy terminal portion of collagen XVIII. Endostatin is a specific inhibitor of endothelial proliferation and angiogenesis. Systemic administration of non-refolded precipitated protein expressed in *Escherichia coli* caused growth regression of Lewis lung carcinoma, T241 fibrosarcoma, B16 melanoma and EOMA (O'Reilly *et al.* (1997) *Cell* 88:277-85) cells in a xenograft model. Moreover, no drug resistance was noted in three of the tumor types studied. Repeated cycles of administration with endostatin have been reported to result in tumor dormancy (Boehm *et al.* (1997) *Nature* 390:404-407).

The results from these studies open new avenues for treatment of cancer and provide promising routes for overcoming the drug resistance often seen during chemotherapy. However, in all of these investigations, a non-refolded precipitated form of the inhibitor protein was administered in the form of a suspension to tumor

bearing animals. In addition, large amounts of protein were required to cause tumor regression and to lead to tumor dormancy. As pointed out by Kerbel ((1997) *Nature* 390:335-36), oral drug equivalents of these proteins are needed. Mechanistic investigations could be undertaken if recombinant forms of these proteins were available in soluble form. Moreover, initial testing could be done *in vitro* with soluble protein before studying its efficacy under *in vivo* conditions. Furthermore, there have been reports that despite the great promise held by these proteins, evaluation of their clinical potential is stymied due to difficulties in producing enough of the protein to test, and inconsistent test results regarding their anti-angiogenic properties (King, R.T. (1998) *Wall Street J.*, page 1 Nov. 12; Leff, D.N. (1998) *BioWorld Today* 9:1, Oct. 20). There clearly exists at the present time a great need for a method of producing soluble forms of anti-angiogenic proteins in large amounts, and which have reliable properties *in vitro* and *in vivo*.

SUMMARY OF THE INVENTION

Restin, a novel anti-angiogenic protein is described, as well as fragments, mutants, derivatives and fusion proteins, thereof. Restin is a proteolytic fragment of the C-terminal fragment of collagen XV, and is approximately 20 kDa. One fragment, designated "apomigren," was found to have anti-angiogenic activity equal or superior to that of endostatin. Methods for expression of the proteins at high titer are also described.

The invention relates to the discovery of an isolated anti-angiogenic peptide, designated "restin," and its fragment, designated "apomigren." Restin comprises about 170 to about 200 amino acid residues, and has at least 70% sequence identity with the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen. Apomigren is a fragment of restin, and comprises the last 80 to 90 contiguous amino acids corresponding to the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen.

The invention also comprises isolated polynucleotides encoding restin and apomigren, complementary sequences and sequences that hybridize those sequences described herein, operably linked to expression sequences, and host cells

transformed with such a construct. Antibodies to restin and apomigren are also disclosed.

The invention also relates to processes for producing restin and apomigren, fusion proteins containing restin and apomigren, and compositions comprising restin and apomigren or fusion products thereof. The invention also discloses methods of producing polypeptides encoding restin and apomigren.

In addition, the invention comprises methods for inhibiting angiogenic activity in mammalian tissue, comprising contacting the tissue with a composition comprising restin and apomigren, particularly to inhibit angiogenesis, which occurs in many diseases and conditions, including cancer.

The invention also discloses use of restin and apomigren to induce apoptosis, or antibodies of EM 1 to prevent apoptosis. The invention further discloses use of restin and apomigren in methods of gene therapy. The cells targeted may be any mammalian cells, particularly lymphocytes, blood cells, TIL cells, bone marrow cells, vascular cells, tumor cells, liver cells, muscle cells, and fibroblast cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram of the restin nucleotide sequence (SEQ ID NO:).

Fig. 2 is a diagram of the restin amino acid sequence (SEQ ID NO:).

Fig. 3 is a schematic diagram of the *Pichia pastoris* expression vector from InVitrogen (San Diego, California, USA).

Fig. 4 is a schematic diagram of the modified *Pichia pastoris* expression vector.

Fig. 5 is a schematic representation of the cloning of restin into the modified *Pichia pastoris* expression system.

Fig. 6 is a SDS-PAGE electrophoresis gel stained with Coomassie Blue, showing protein expression from the various libraries.

Fig. 7 consists of four plots of the hydrophilicity, surface probability, flexibility, and antigenic index of the restin protein.

Fig. 8 consists of three plots showing the amphiphilic helix, amphiphilic sheet, and secondary structure of the restin protein.

Fig. 9 is a schematic presentation of the full length α1 chain of human collagen type XV in context with the C-terminal 85-amino acid protein apomigren. Dark black boxes indicate collagenous regions and the white boxes, non-collagenous domains.

Fig. 10 is an alignment of the amino acid sequences of apomigren, and the C-terminus of mouse and human endostatin.

Fig. 11 is a graph showing the elution profile of recombinant apomigren from the Ni-NTA column. The elution pH (left y-axis) and absorbance at 280 nM (right y-axis) are shown for each fraction (x-axis).

Fig. 12 is a photograph of an SDS-PAGE gel. Sizes in kDa are shown on the left. Lane 1 contains size marker, lane 2, apomigren protein lysate prior to loading on the Ni-NTA column; lane 2, flowthrough from the column; lane 4, fraction 4; lanes 5 and 6, fraction 23, non-reduced and reduced, respectively; lane 7, fraction 24; lane 8, fraction 27.

Figs. 13A, 13B and 13C are a set of three photographs showing the changes in C-PAE cell morphology following apomigren treatment as monitored by phase contrast microscopy. Fig. 13A, control; Fig. 13B, treatment with 0.05 μg/ml apomigren; Fig. 13C, treatment with 0.7 μg/ml apomigren. Pictures were taken 24 hours after treatment, and are shown at 400X magnification.

Fig. 14 is a graph showing thymidine incorporation into C-PAE cells treated with bFGF and purified soluble apomigren protein expressed from bacteria. Concentration of apomigren (●), and mouse (□) and human (○) endostatin are shown on the x-axis, and ^{3}H-thymidine incorporation is on the y-axis.

Fig. 15 is a bar graph showing cell cycle analysis with apomigren. The x-axis shows concentration of apomigren, ranging from 0 to 1000 ng/ml, and the y-axis shows percentage of cells in S-phase. C-PAE cells are represented by dark bars and IMR-90 cells by white bars.

Fig. 16 is a graph showing inhibition of endothelial cell migration with different concentrations of apomigren, and mouse and human endostatin. The concentration of the various proteins is on the x-axis and percent cells that migrated in response to bFGF on the y-axis. The control is apomigren-treated IC-21 cells (■),

and treatments consist of apomigren (●) and mouse (□) and human (○) endostatin on ECV304 cells.

Figure 17 is a bar graph showing inhibition of all migration in ECV304 cell traeted with restin. Amount of cell migration is shown on the y-axis, and the x-axis shows ECV304 cells treated with either nothing (-bFGF, control), bFGF (positive control), and restin at 3.0, 62.5, 12, 25 and 50 μg/ml.

Figure 18 is a graph showing the effects of restin on renal cancer cell (RCC) tumor growth, as compared to endostatin. Tumor volume is mm^3 is sown on the y-axis, plotted against days after treatment (x-axis). Restin (●) compared favorably with endostatin (○) in slowing tumor growth, relative to untreated control tumors (■).

Figure 19 is a bar graph showing the effects of varying concentrations of apomigren (x-axis) on angiogenic response (y-axis) as measured by the chorioallantoic membrane (CAM) assay. Apomigren (0.5, 1, 5, 10, 20 and 50 μg/mesh) was tested against vehicle alone, and VEGF and FGF-2.

Figure 20A-B is a chart showing the constructs, primers, cloning sites, and vectors, used to clone and express various anti-angiogenic proteins. The amino acid sequences of the expressed proteins are also given.

DETAILED DESCRIPTION OF THE INVENTION

A wide variety of diseases are the result of undesirable angiogenesis. Put another way, many diseases and undesirable conditions could be prevented or alleviated if it were possible to stop the growth and extension of capillary blood vessels under some conditions, at certain times, or in particular tissues. Several anti-angiogenic proteins have been discovered (*e.g.*, angiostatin, endostatin), but problems have been reported regarding (1) the ability to produce the proteins in sufficient quantity to allow for proper testing of their properties, and (2) the reproducibility of the anti-angiogenic properties attributed to these proteins.

In the present invention, restin, a novel anti-angiogenic protein, is described, as well as fragments, fusion proteins and antibodies thereof. Polynucleotides encoding restin and its fragments are also described, a well as vectors and host cells

comprising those polynucleotides. Compositions containing restin as a biologically active component are also described, as well as methods for using restin to inhibit angiogenic activity in mammalian tissues, such as in treating diseases and conditions characterized by angiogenesis.

The present invention includes compositions and methods for the detection and treatment of diseases and conditions that are mediated by or associated with angiogenesis. Unlike other anti-angiogenic proteins, (*e.g.*, angiostatin, endostatin), restin is a proteolytic fragment of about 170 to about 200 amino acids, corresponding to the approximately 200 extreme C-terminal amino acids of the NC10 domain of the α1 chain of human Type XV collagen. Unlike endostatin, restin has no or minimal affinity for heparin.

More particularly, the invention describes a protein designated "restin," which is a protein of about 170 amino acids to about 200 amino acids, corresponding to the approximately 200 amino acids at the C-terminus of the α1 chain of the NC10 domain of human Type XV collagen. Unlike angiostatin and endostatin, restin has not been successfully isolated from body fluids, and appears to not exist naturally outside of the native collagen XV molecule. Restin can be cloned out of isolated DNA or a cDNA library. Restin has a molecular weight of about 20 kDa as determined by reducing polyacrylamide gel electrophoresis. Also encompassed by the present invention is mouse restin, fragments, mutants, derivatives or fusion proteins thereof.

Restin exists naturally as part of the collagen Type XV molecule, but it can be produced recombinantly, *e.g.*, the polynucleotide sequence (Fig. 1, SEQ ID NO:) encoding restin (Fig. 2, SEQ ID NO:) can amplified, *e.g.*, with the forward and reverse primers listed in Table 1, below. The template nucleic acid used for the amplification can be from any mammal.

Table 1. Constructs and primer sequences used to amplify anti-angiogenic proteins.

Construct Name	Primer Sequence
pET17bhis.mendo	5'-GGC ATA TGC ATA CTC ATC AGG ACT TT-3' (up) (SEQ ID NO.:)
	5' AAC TCG AGC TAT TTG GAG AAA GAG GT-3' (down) (SEQ ID NO.:)
pET28a/mendo	5'-GGC ATA TGC ATA CTC ATC AGG ACT TT-3' (up)
	5'-AAG CGG CCG CCT ATT TGG AGA AAG AGG T-3' (down) (SEQ ID NO:)
pET28a/EM-1	5' TTC CAT ATG CAT ACT CAT CAG GAC TTT CAG CCA-3' (up) (SEQ ID NO:)
	5' TTA GCG GCC GCC TAC TCA ATG CAC AGG ACG ATG TA-3' (down) (SEQ ID NO:)
pET28a/EM-2	5' TTC CAT ATG CAT ACT CAT CAG GAC TTT CAG CCA-3' (up) (SEQ ID NO:)
	5' TTA GCG GCC GCC TAG TTG TGG CAG CTC GCA GCT TTC TG-3' (down) (SEQ ID NO:)
pPICZαA/mendo	5' GGG AAT TCC ATA CTC ATC AGG ACT TT-3' (up) (SEQ ID NO:)
	5' AAG CGG CCG CCT ATT TGG AGA AAG AGG T-3' (down) (SEQ ID NO:)
pPICZαA/His.mendo	5' AAG AAT TCC ATC ATC ATC ATC ATC ACA GCA GC-3' (up) (SEQ ID NO:)
	5' AAG CGG CCG CCT ATT TGG AGA AAG AGG T-3' (down) (SEQ ID NO:)
pPICZαA/Hendo	5' TTT GAA TTC GCC CAC AGC CAC CGC GAC TTC CAG CCG GTG CTC CA-3' (up) (SEQ ID NO:)

	5' AAA AGC GGC CGC CTA CTT GGA GGC AGT CAT GAA GCT GTT CTC AA-3' (down) (SEQ ID NO:)
pPICZαA/Restin	5' TTT TTT GAA TTC ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT-3' (up) (SEQ ID NO:)
	5' AAG AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TCG AT-3' (down) (SEQ ID NO:)
pPICZαA/HIS.Restin	5' AAT TCC ATC ACC ATC ACC ATC ACG-3' (up) (SEQ ID NO:)
	5' AAT TCG TGA TGG TGA TGG TGA TGG-3' (down) (SEQ ID NO:)
pET28a/M2	5' TTT CAT ATG ATA TAC TCC TTT GAT GGT CGA GAC ATA ATG ACA-3' (up) (SEQ ID NO:)
	5' AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TCG AT-3' (down) (SEQ ID NO:)
pPICZαA/M2	5' AAG AAT TCC ATC ATC ATC ATC ATC ACA GCA GC-3' (up) (SEQ ID NO:)
	5' AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TCG AT-3' (down) (SEQ ID NO:)

The resulting polynucleotide amplification product can then be cloned into a suitable vector. The term "primer" denotes a specific oligonucleotide sequence complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence and serve as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase. "Restin," as used herein, is intended to include fragments, mutants, homologs, analogs, and allelic variants of the amino acid sequence of (SEQ ID NO:) as well as mouse restin, fragments, mutants, homologs, analogs and allelic variants of the mouse restin amino acid sequence.

It is to be understood that the present invention is contemplated to include any derivatives of the restin that have endothelial inhibitory activity (*e.g.*, the capability of a composition to inhibit angiogenesis in general and, for example, to inhibit the growth or migration of bovine capillary endothelial cells in culture in the presence of fibroblast growth factor, angiogenesis-associated factors, or other known growth factors). The present invention includes the entire restin protein, derivatives of the restin protein and biologically-active fragments of the restin protein. These include proteins with restin activity that have amino acid substitutions or have sugars or other molecules attached to amino acid functional groups. The present invention also includes genes that code for restin and the restin receptor, and to proteins that are expressed by those genes.

The invention also encompasses a composition comprising an isolated polynucleotide encoding restin, as well as vectors and host cells containing such a polynucleotide, and processes for producing restin and its fragments, mutants, homologs, analogs and allelic variants. The term "vector" as used herein means a carrier into which pieces of nucleic acid may be inserted or cloned, which carrier functions to transfer the pieces of nucleic acid into a host cell. Such a vector may also bring about the replication and/or expression of the transferred nucleic acid pieces. Examples of vectors include nucleic acid molecules derived, *e.g.*, from a plasmid, bacteriophage, or mammalian, plant or insect virus, or non-viral vectors such as ligand-nucleic acid conjugates, liposomes, or lipid-nucleic acid complexes. It may be desirable that the transferred nucleic molecule is operatively linked to an expression control sequence to form an expression vector capable of expressing the transferred nucleic acid. Such transfer of nucleic acids is generally called "transformation," and refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion. For example, direct uptake, transduction or f-mating are included. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. "Operably linked" refers to a situation wherein the components described are in a relationship permitting them to function in their intended manner, *e.g.*, a control sequence "operably linked" to a coding

sequence is ligated in such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequence. A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of (*e.g.*, operably linked to) appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. Such boundaries can be naturally-occurring, or can be introduced into or added the polynucleotide sequence by methods known in the art. A coding sequence can include, but is not limited to, mRNA, cDNA, and recombinant polynucleotide sequences.

The vector into which the cloned polynucleotide is cloned may be chosen because it functions in a prokaryotic, or alternatively, it is chosen because it functions in a eukaryotic organism. Two examples of vectors which allow for both the cloning of a polynucleotide encoding the restin protein, and the expression of that protein from the polynucleotide, are the pET28(a) vector (Novagen, Madison, Wisconsin, USA) and a modified pPICZαA vector (InVitrogen, San Diego, California, USA), which allow expression of the protein in bacteria and yeast, respectively.

Once a polynucleotide has been cloned into a suitable vector, it can be transformed into an appropriate host cell. By "host cell" is meant a cell which has been or can be used as the recipient of transferred nucleic acid by means of a vector. Host cells can prokaryotic or eukaryotic, mammalian, plant, or insect, and can exist as single cells, or as a collection, *e.g.*, as a culture, or in a tissue culture, or in a tissue or an organism. Host cells can also be derived from normal or diseased tissue from a multicellular organism, *e.g.*, a mammal. Host cell, as used herein, is intended to include not only the original cell which was transformed with a nucleic acid, but also descendants of such a cell, which still contain the nucleic acid.

In one embodiment, the isolated polynucleotide encoding the anti-angiogenic protein additionally comprises a polynucleotide linker encoding a peptide. Such linkers are known to those of skill in the art and, for example the linker can comprise at least one additional codon encoding at least one additional amino acid. Typically

the linker comprises one to about twenty or thirty amino acids. The polynucleotide linker is translated, as is the polynucleotide encoding the anti-angiogenic protein, resulting in the expression of an anti-angiogenic protein with at least one additional amino acid residue at the amino or carboxyl terminus of the anti-angiogenic protein. Some linkers attached to anti-angiogenic proteins are illustrated in Figure 20. Importantly, the additional amino acid, or amino acids, do not compromise the activity of the anti-angiogenic protein.

After inserting the selected polynucleotide into the vector, the vector is transformed into an appropriate prokaryotic strain and the strain is cultured (*e.g.*, maintained) under suitable culture conditions for the production of the biologically active anti-angiogenic protein, thereby producing a biologically active anti-angiogenic protein, or mutant, derivative, fragment or fusion protein thereof. In one embodiment, the invention comprises cloning of a polynucleotide encoding an anti-angiogenic protein into the vectors pET17b or pET28a, which are then transformed into bacteria. The bacterial host strain then expresses the anti-angiogenic protein. Typically the anti-angiogenic proteins are produced in quantities of about 10-20 milligrams, or more, per liter of culture fluid.

In another embodiment of the present invention, the eukaryotic vector comprises a modified yeast vector. As described herein, one method uses a pPICzα plasmid wherein the plasmid contains a multiple cloning site. The multiple cloning site has inserted into the multiple cloning site a His.Tag motif. Additionally the vector can be modified to add a *Nde*I site, or other suitable restriction sites. Such sites are well known to those of skill in the art. Anti-angiogenic proteins produced by this embodiment comprise a histidine tag motif (His.tag) comprising one, or more histidines, typically about 5-20 histidines. Surprisingly, this His.tag does not compromise anti-angiogenic activity.

In this embodiment, a preferred yeast expression system is *Pichia pastores*. Again, the biologically active protein is typically produced at concentrations of about 10-20 milligrams per liter of culture medium (fluid).

One method of producing restin, for example, is to amplify the polynucleotide of SEQ ID NO: , clone it into an expression vector, *e.g.*, pET28(a), pPICZαA, or some other expression vector, transform the vector containing the polynucleotide of SEQ ID NO: into a host cell capable of expressing the polypeptide encoded by the polynucleotide, culturing the transformed host cell under culture conditions suitable for expressing the protein, and then extracting and purifying the protein from the culture. Exemplary methods of producing anti-angiogenic proteins in general, and restin in particular, are provided in the Examples below, and also in U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-Angiogenic Proteins," by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of all of which are herein incorporated by reference. The restin protein may also be expressed as a product of transgenic animals, *e.g.*, as a component of the milk of transgenic cows, goats, sheep or pigs, or as a product of a transgenic plant, *e.g.*, combined or linked with starch molecules in maize.

Restin may also be produced by conventional, known methods of chemical synthesis. Methods for constructing the proteins of the present invention by synthetic means are known to those skilled in the art. The synthetically-constructed restin protein sequences, by virtue of sharing primary, secondary or tertiary structural and/or conformational characteristics with *e.g.*, recombinantly-produced restin, may possess biological properties in common therewith, including biological activity. Thus, the synthetically-constructed restin protein sequences may be employed as biologically active or immunological substitutes for *e.g.*, recombinantly-produced, purified restin protein in screening of therapeutic compounds and in immunological processes for the development of antibodies.

The restin protein is useful in inhibiting angiogenesis, as determined in standard assays, and provided in the Examples below. Restin does not inhibit the growth of other cells types, *e.g.*, IMR-90 cells, or IC-21 cells. Tests have shown that in endothelial cell migration assays with ECV304 cells, treatment with restin resulted in 50% inhibition at levels of 2.0 - 5.0 μg/ml, *e.g.*, restin has an ED_{50} of about 2.0 - 5.0 μg/ml. This is similar to that of endostatin, but inferior to the activity found for apomigren. In *in vivo* studies (*e.g.*, a renal cancer cell (RCC) model),

human restin treatment resulted in 20-50% inhibition of tumor growth. Mouse endostatin performed better, presumably due to the better "fit" of mouse protein on mouse vasculature. As used herein, "ED_{50}" is an abbreviation for the amount of a composition which reduces a biological effect by one-half, relative to the biological effect seen in the absence of the composition.

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ, and involves endothelial cell proliferation. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development, and formation of the corpus luteum, endometrium and placenta. The term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels, and blood vessels. "Anti-angiogenic activity" therefore refers to the capability of a composition to inhibit the growth of blood vessels. The growth of blood vessels is a complex series of events, and includes localized breakdown of the basement membrane lying under the individual endothelial cells, proliferation of those cells, migration of the cells to the location of the future blood vessel, reorganization of the cells to form a new vessel membrane, cessation of endothelial cell proliferation, and, incorporation of pericytes and other cells that support the new blood vessel wall. "Anti-angiogenic activity" as used herein therefore includes interruption of any or all of these stages, with the end result that formation of new blood vessels is inhibited.

Anti-angiogenic activity may include endothelial inhibiting activity, which refers to the capability of a composition to inhibit angiogenesis in general and, for example, to inhibit the growth or migration of bovine capillary endothelial cells in culture in the presence of fibroblast growth factor, angiogenesis-associated factors, or other known growth factors. A "growth factor" is a composition that stimulates the growth, reproduction, or synthetic activity of cells. An "angiogenesis-associated factor" is a factor which either inhibits or promotes angiogenesis. An example of an angiogenesis-associated factor is an angiogenic growth factor, such as basic fibroblastic growth factor (bFGF), which is an angiogenesis promoter. Another example of an angiogenesis-associated factor is an angiogenesis inhibiting factor

such as *e.g.*, angiostatin (see, *e.g.*, U.S. Pat. No. 5,801,012, U.S. Pat. No. 5,837,682, U.S. Pat. No. 5,733,876, U.S. Pat. No. 5,776,704, U.S. Pat. No. 5,639,725, U.S. Pat. No. 5,792,845, WO 96/35774, WO 95/29242, WO 96/41194, WO 97/23500) or endostatin (see, *e.g.*, WO 97/15666).

By "substantially the same biological activity" or "substantially the same or superior biological activity" is meant that a composition has anti-angiogenic activity, and behaves similarly as does restin, as determined in standard assays. "Standard assays" include, but are not limited to, those protocols used in the molecular biological arts to assess anti-angiogenic activity, cell cycle arrest, and apoptosis. Such assays include, but are not limited to, assays of endothelial cell proliferation, endothelial cell migration, cell cycle analysis, and endothelial cell tube formation, detection of apoptosis, *e.g.*, by apoptotic cell morphology or Annexin V-FITC assay, chorioallantoic membrane (CAM) assay, and inhibition of renal cancer tumor growth in nude mice. Such assays are provided in the Examples below, and in U.S.S.N. 60/067,888, filed December 8, 1997, U.S.S.N. 60/082,663, filed April 22, 1998, U.S.S.N. 60/108,536, filed November 16, 1998, and in U.S.S.N. XX/XXX,XXX, "Anti-Angiogenic Peptides and Methods of Use Thereof," by Vikas P. Sukhatme, filed December 8, 1998, and U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-Angiogenic Proteins," by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of all of which are herein incorporated by reference.

The invention also describes fragments, mutants, homologs and analogs of restin. A "fragment" of restin is any amino acid sequence shorter that the restin molecule, comprising at least 25 consecutive amino acids of the restin polypeptide. Such mutants may or may not also comprise additional amino acids derived from the process of cloning, *e.g.*, amino acid residues or amino acid sequences corresponding to full or partial linker sequences. To be encompassed by the present invention, such mutants, with or without such additional amino acid residues, must have substantially the same biological activity as the natural or full-length version of the reference polypeptide.

One such fragment, designated "apomigren", was found to have anti-angiogenic activity equivalent or superior to that of human or mouse endostatin, as

determined by standard assays. apomigren comprises the last approximately 85 amino acid residues of restin itself, from about amino acid 97 to about amino acid 181 of SEQ ID NO: :

IYS FDG RDI MTD PSW PQK VIW HGS SPH GVR LVD NYC EAW RTA DTA VTG LAS PLS TGK ILD QKA YSC ANR LIV LCI ENS FMT DAR K

The polynucleotide sequence encoding apomigren therefore corresponds to about nucleotide 289 to about nucleotide 543 of SEQ ID NO: , and can be amplified out of SEQ ID NO: with the forward primer 5'-AAG AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TCG AT-3' and the same reverse primer as that used for restin. Cloning and expression of apomigren is done as for restin itself, as illustrated in the Examples below. Apomigren provides an advantage in treatment of angiogenic diseases in that increasingly smaller peptides are more potent on a weight basis, and may be able to better penetrate tissues.

By "mutant" of restin is meant a polypeptide that includes any change in the amino acid sequence relative to the amino acid sequence of the equivalent reference restin polypeptide. Such changes can arise either spontaneously or by manipulations by man, by chemical energy (*e.g.*, X-ray), or by other forms of chemical mutagenesis, or by genetic engineering, or as a result of mating or other forms of exchange of genetic information. Mutations include, *e.g.*, base changes, deletions, insertions, inversions, translocations, or duplications. Mutant forms of restin may display either increased or decreased anti-angiogenic activity relative to the equivalent reference restin polynucleotide, and such mutants may or may not also comprise additional amino acids derived from the process of cloning, *e.g.*, amino acid residues or amino acid sequences corresponding to full or partial linker sequences.

By "analog" of restin is meant a non-natural molecule substantially similar to either the entire restin molecule or a fragment or allelic variant thereof, and having substantially the same or superior biological activity. Such analogs are intended to include derivatives (*e.g.*, chemical derivatives, as defined above) of the biologically active restin, as well as its fragments, mutants, homologs, and allelic variants, which

derivatives exhibit a qualitatively similar agonist or antagonist effect to that of the unmodified restin polypeptide, fragment, mutant, homolog, or allelic variant.

By "allele" of restin is meant a polypeptide sequence containing a naturally-occurring sequence variation relative to the polypeptide sequence of the reference restin polypeptide. By "allele" of a polynucleotide encoding the restin polypeptide is meant a polynucleotide containing a sequence variation relative to the reference polynucleotide sequence encoding the reference restin polypeptide, where the allele of the polynucleotide encoding the restin polypeptide encodes an allelic form of the restin polypeptide.

It is possible that a given polypeptide may be either a fragment, a mutant, an analog, or allelic variant of restin, or it may be two or more of those things, *e.g.*, a polypeptide may be both an analog and a mutant of the restin polypeptide. For example, a shortened version of the restin molecule (*e.g.*, a fragment of restin) may be created in the laboratory. If that fragment is then mutated through means known in the art, a molecule is created that is both a fragment and a mutant of restin. In another example, a mutant of restin may be created, which is later discovered to exist as an allelic of restin in some mammalian individuals. Such a mutant restin molecule would therefore be both a mutant and an allelic variant of restin. Such combinations of fragments, mutants, allelic variants, and analogs are intended to be encompassed in the present invention.

Encompassed by the present invention are proteins that have substantially the same amino acid sequence as restin or apomigren, or polynucleotides that have substantially the same nucleic acid sequence as the polynucleotides encoding restin or apomigren. "Substantially the same sequence" means a nucleic acid or polypeptide that exhibits at least about 70 % sequence identity with a reference sequence, *e.g.*, another nucleic acid or polypeptide, typically at least about 80% sequence identity with the reference sequence, preferably at least about 90% sequence identity, more preferably at least about 95% identity, and most preferably at least about 97% sequence identity with the reference sequence. The length of comparison for sequences will generally be at least 75 nucleotide bases or 25 amino acids, more preferably at least 150 nucleotide bases or 50 amino acids, and most

preferably 243-264 nucleotide bases or 81-88 amino acids. "Polypeptide" as used herein indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to include polypeptide that have been subjected to post-expression modifications such as, for example, glycosylations, acetylations, phosphorylations and the like. Restin, in general, has less than 70% amino acid sequence identity with endostatin.

"Sequence identity," as used herein, refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, two polynucleotides or two polypeptides . When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two peptides is occupied by serine, then they are identical at that position. The identity between two sequences is a direct function of the number of matching or identical positions, *e.g.*, if half (*e.g.*, 5 positions in a polymer 10 subunits in length), of the positions in two peptide or compound sequences are identical, then the two sequences are 50% identical; if 90% of the positions, *e.g.*, 9 of 10 are matched, the two sequences share 90% sequence identity. By way of example, the amino acid sequences VRGLQP and HAFLQP have 3 of 6 positions in common, and therefore share 50% sequence identity, while the sequences VRGLQP and AFLQP have 3 of 5 positions in common, and therefore share 60% sequence identity. The identity between two sequences is a direct function of the number of matching or identical positions. Thus, if a portion of the reference sequence is deleted in a particular peptide, that deleted section is not counted for purposes of calculating sequence identity, *e.g.*, VRGLQP and VRGLP have 5 out of 6 position in common, and therefore share 83.3% sequence identity.

Identity is often measured using sequence analysis software *e.g.*, BLASTN or BLASTP (available at http://www.ncbi.nlm.nih.gov/BLAST/). The default parameters for comparing two sequences (*e.g.*, "Blast"-ing two sequences against each other, http://www.ncbi.nlm.nih.gov/gorf/bl2.html) by BLASTN (for nucleotide sequences) are reward for match = 1, penalty for mismatch = -2, open gap = 5, extension gap = 2. When using BLASTP for protein sequences, the default

parameters are reward for match = 0, penalty for mismatch = 0, open gap = 11, and extension gap = 1.

When two sequences share "sequence homology," it is meant that the two sequences differ from each other only by conservative substitutions. For polypeptide sequences, such conservative substitutions consist of substitution of one amino acid at a given position in the sequence for another amino acid of the same class (*e.g.*, amino acids that share characteristics of hydrophobicity, charge, pK or other conformational or chemical properties, *e.g.*, valine for leucine, arginine for lysine), or by one or more non-conservative amino acid substitutions, deletions, or insertions, located at positions of the sequence that do not alter the conformation or folding of the polypeptide to the extent that the biological activity of the polypeptide is destroyed. Examples of "conservative substitutions" include substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine; the substitution of one basic residue such as lysine, arginine or histidine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another; or the use of a chemically derivatized residue in place of a non-derivatized residue; provided that the polypeptide displays the requisite biological activity. Two sequences which share sequence homology may called "sequence homologs."

Homology, for polypeptides, is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Protein analysis software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine threonine; lysine, arginine; and phenylalanine, tyrosine.

Also encompassed by the present invention are chemical derivatives of restin and apomigren. "Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized residues include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-imbenzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substitute for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine.

Polynucleotides encoding restin can be cloned out of isolated DNA or a cDNA library. Nucleic acids polypeptides, referred to herein as "isolated" are nucleic acids or polypeptides substantially free (*i.e.*, separated away from) the material of the biological source from which they were obtained (*e.g.*, as exists in a mixture of nucleic acids or in cells), which may have undergone further processing. "Isolated" nucleic acids or polypeptides include nucleic acids or polypeptides obtained by methods described herein, similar methods, or other suitable methods, including essentially pure nucleic acids or polypeptides, nucleic acids or polypeptides produced by chemical synthesis, by combinations of chemical or biological methods, and recombinantly produced nucleic acids or polypeptides which are isolated. An isolated polypeptide therefore means one which is relatively free of other proteins, carbohydrates, lipids, and other cellular components with which it is normally associated. An isolated nucleic acid is not immediately contiguous with (*i.e.*, covalently linked to) both of the nucleic acids with which it is immediately contiguous in the naturally-occurring genome of the organism from which the nucleic acid is derived. The term, therefore, includes, for example, a

nucleic acid which is incorporated into a vector (*e.g.*, an autonomously replicating virus or plasmid), or a nucleic acid which exists as a separate molecule independent of other nucleic acids such as a nucleic acid fragment produced by chemical means or restriction endonuclease treatment.

The polynucleotides and proteins of the present invention can also be used to design probes to isolate other anti-angiogenic proteins. Exceptional methods are provided in U.S. Pat. No. 5,837,490, by Jacobs *et al.*, the entire teachings of which are herein incorporated by reference in their entirety. The design of the oligonucleotide probe should preferably follow these parameters: (a) It should be designed to an area of the sequence which has the fewest ambiguous bases ("N's"), if any, and (b) It should be designed to have a T_m of approx. 80° C (assuming 2°C for each A or T and 4 degrees for each G or C).

The oligonucleotide should preferably be labeled with g-^{32}P ATP (specific activity 6000 Ci/mmole) and T4 polynucleotide kinase using commonly employed techniques for labeling oligonucleotides. Other labeling techniques can also be used. Unincorporated label should preferably be removed by gel filtration chromatography or other established methods. The amount of radioactivity incorporated into the probe should be quantitated by measurement in a scintillation counter. Preferably, specific activity of the resulting probe should be approximately 4 x 10^6 dpm/pmole. The bacterial culture containing the pool of full-length clones should preferably be thawed and 100 µl of the stock used to inoculate a sterile culture flask containing 25 ml of sterile L-broth containing ampicillin at 100 µg/ml. The culture should preferably be grown to saturation at 37°C, and the saturated culture should preferably be diluted in fresh L-broth. Aliquots of these dilutions should preferably be plated to determine the dilution and volume which will yield approximately 5000 distinct and well-separated colonies on solid bacteriological media containing L-broth containing ampicillin at 100 µg/ml and agar at 1.5% in a 150 mm petri dish when grown overnight at 37°C. Other known methods of obtaining distinct, well-separated colonies can also be employed.

Standard colony hybridization procedures should then be used to transfer the colonies to nitrocellulose filters and lyse, denature and bake them. Highly stringent

condition are those that are at least as stringent as, for example, 1x SSC at 65°C, or 1x SSC and 50% formamide at 42°C. Moderate stringency conditions are those that are at least as stringent as 4x SSC at 65°C, or 4x SSC and 50% formamide at 42°C. Reduced stringency conditions are those that are at least as stringent as 4x SSC at 50°C, or 6x SSC and 50% formamide at 40°C.

The filter is then preferably incubated at 65°C for 1 hour with gentle agitation in 6.times. SSC (20x stock is 175.3 g NaCl/liter, 88.2 g Na citrate/liter, adjusted to pH 7.0 with NaOH) containing 0.5% SDS, 100 μg/ml of yeast RNA, and 10 mM EDTA (approximately 10 mL per 150 mm filter). Preferably, the probe is then added to the hybridization mix at a concentration greater than or equal to 1 x 10^6 dpm/mL. The filter is then preferably incubated at 65°C with gentle agitation overnight. The filter is then preferably washed in 500 mL of 2x SSC/0.5% SDS at room temperature without agitation, preferably followed by 500 mL of 2x SSC/0.1% SDS at room temperature with gentle shaking for 15 minutes. A third wash with 0.1x SSC/0.5% SDS at 65°C for 30 minutes to 1 hour is optional. The filter is then preferably dried and subjected to autoradiography for sufficient time to visualize the positives on the X-ray film. Other known hybridization methods can also be employed. The positive colonies are then picked, grown in culture, and plasmid DNA isolated using standard procedures. The clones can then be verified by restriction analysis, hybridization analysis, or DNA sequencing.

The present invention also includes fusion proteins and chimeric proteins comprising restin, its fragments, mutants, homologs, analogs, and allelic variants. A fusion or chimeric protein can consist of a multimer of a single protein, *e.g.*, repeats of restin or repeats of apomigren, or the fusion and chimeric proteins can be made up of several proteins, *e.g.*, restin and apomigren. The fusion proteins can comprise a combination of two or more known anti-angiogenic proteins (*e.g.*, angiostatin and endostatin, or biologically active fragments of angiostatin and endostatin), or an anti-angiogenic protein in combination with a targeting agent (*e.g.*, endostatin with epidermal growth factor (EGF) or RGD peptides), or an anti-angiogenic protein in combination with an immunoglobulin molecule (*e.g.*, endostatin and IgG, specifically with the Fc portion removed). The fusion and chimeric proteins can also

include restin, apomigren, their fragments, mutants, homologs, analogs, and allelic variants, and other anti-angiogenic proteins, *e.g.*, endostatin, angiostatin, or EM 1 (as described in U.S.S.N. XX/XXX,XXX, "Anti-Angiogenic Peptides and Methods of Use Thereof", by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of which are herein incorporated by reference). The term "fusion protein" as used herein can also encompass additional components for e.g., delivering a chemotherapeutic agent, wherein a polynucleotide encoding the chemotherapeutic agent is linked to the polynucleotide encoding the anti-angiogenic protein. Fusion proteins can also encompass multimers of the anti-angiogenic protein, *e.g.*, a dimer or trimer of endostatin. Such fusion proteins can be linked together via post-translational modification (*e.g.*, chemically linked), or the entire fusion protein may be made recombinantly.

Also included in the inventions are compositions containing, as a biological ingredient, restin, as well as its fragments, mutants, homologs, analogs, and allelic variants to inhibit or enhance angiogenesis in mammalian tissues, and use of such compositions in the diagnosis, prognosis, and treatment of diseases and conditions characterized by, or associated with, angiogenic activity or lack thereof. Such methods can involve administration by oral, topical, injection, implantation, sustained release, or other delivery methods.

The invention includes use of restin, and its fragments, mutants, homologs, analogs, allelic variants, and fusion and chimeric proteins as biologically-active agents in compositions for the purpose of treating diseases or conditions that are associated with angiogenic activity. Methods of treating such diseases include contacting the affected tissue with a composition comprising restin, its fragments, mutants, homologs, analogs, or allelic variants.

The present invention includes the method of treating an angiogenesis-mediated disease with a therapeutically effective amount of restin, or a biologically active fragment thereof, or combinations of restin fragments that possess anti-angiogenic activity, or restin agonists and antagonists. Angiogenesis-mediated diseases include, but are not limited to, cancers, solid tumors, blood-born tumors (*e.g.*, leukemias), tumor metastasis, benign tumors (*e.g.*, hemangiomas, acoustic

neuromas, neurofibromas, trachomas, and pyogenic granulomas), rheumatoid arthritis, psoriasis, ocular angiogenic diseases (*e.g.*, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis), Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, and wound granulation. Restin is useful in the treatment of diseases of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, Crohn's disease, atherosclerosis, scleroderma, and hypertrophic scars (*i.e.*, keloids). Restin can be used as a birth control agent by preventing vascularization required for embryo implantation. Restin is useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochele minalia quintosa*) and ulcers (*Heliobacter pylori*). Restin and apomigren can also be used to prevent dialysis graft vascular access stenosis, and obesity, *e.g.*, by inhibiting capillary formation in adipose tissue, thereby preventing its expansion. Restin and apomigren can also be used to treat localized (*e.g.*, nonmetastisized) diseases. "Cancer" means neoplastic growth, hyperplastic or proliferative growth or a pathological state of abnormal cellular development and includes solid tumors, non-solid tumors, and any abnormal cellular proliferation, such as that seen in leukemia. As used herein, "cancer" also means angiogenesis-dependent cancers and tumors, *i.e.*, tumors that require for their growth (expansion in volume and/or mass) an increase in the number and density of the blood vessels supplying them with blood. "Regression" refers to the reduction of tumor mass and size. As used herein, the term "therapeutically effective amount" means the total amount of each active component of the composition or method that is sufficient to show a meaningful patient benefit, *i.e.*, treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

Alternatively, where an increase in angiogenesis is desired, *e.g.*, in wound healing, or in post-infarct heart tissue, antibodies or antisera to the restin protein can be used to block localized, native anti-angiogenic proteins and processes, and thereby increase formation of new blood vessels so as to inhibit atrophy of tissue.

Restin may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation, chemotherapy, or immunotherapy, combined with restin and then restin may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor. Restin or apomigren, or fragments, antisera, receptor agonists, or receptor antagonists thereof, or combinations thereof, can also be combined with other anti-angiogenic compounds, or proteins, fragments, antisera, receptor agonists, receptor antagonists of other anti-angiogenic proteins (*e.g.*, angiostatin, endostatin, EM 1). Additionally, restin, restin fragments, restin antisera, restin receptor agonists, restin receptor antagonists, or combinations thereof, are combined with pharmaceutically acceptable excipients, and optionally sustained-release matrix, such as biodegradable polymers, to form therapeutic compositions. The compositions of the present invention may also contain other anti-angiogenic proteins or chemical compounds, such as endostatin, angiostatin, EM 1 (as described in U.S.S.N. XX/XXX,XXX, "Anti-Angiogenic Peptides and Methods of Use Thereof", by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of which are herein incorporated by reference), and mutants, fragments, and analogs thereof. The compositions may further contain other agents which either enhance the activity of the protein or compliment its activity or use in treatment, such as chemotherapeutic or radioactive agents. Such additional factors and/or agents may be included in the composition to produce a synergistic effect with protein of the invention, or to minimize side effects. Additionally, administration of the composition of the present invention may be administered concurrently with other therapies, *e.g.*, administered in conjunction with a chemotherapy or radiation therapy regimen.

The invention includes methods for inhibiting angiogenesis in mammalian tissues by contacting the tissue with a composition comprising the proteins of the

invention. By "contacting" is meant not only topical application, but also those modes of delivery that introduce the composition into the tissues, or into the cells of the tissues.

Use of timed release or sustained release delivery systems are also included in the invention. Such systems are highly desirable in situations where surgery is difficult or impossible, *e.g.*, patients debilitated by age or the disease course itself, or where the risk-benefit analysis dictates control over cure.

A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polyproteins, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid).

The angiogenesis-modulating composition of the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid compositions include pills, creams, and implantable dosage units. The pills may be administered orally, the therapeutic creams may be administered topically. The implantable dosage unit may be administered locally, for example at a tumor site, or which may be implanted for systemic release of the angiogenesis-modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and intraocular administration. Examples of aerosol formulation include inhaler formulation for administration to the lungs.

The restin proteins and protein fragments with the anti-angiogenic activity described above can be provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (*e.g.*, intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the restin may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the restin is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of restin through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.* (1991) (*J. Neurosurg.* 74:441-446), which is hereby incorporated by reference in its entirety.

The compositions containing a polypeptide of this invention can be administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; *i.e.*, carrier or vehicle.

Modes of administration of the compositions of the present inventions include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. Pharmaceutical compositions for parenteral injection comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles

include water, ethanol, polyois (*e.g.*, glycerol, propylene glycol, polyethylene glycol and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (*e.g.*, olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol sorbic acid and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents, such as aluminum monostearate and gelatin, which delay absorption. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide, poly(orthoesters) and poly(anhydrides). Depending upon the ratio of drug to polymer and the nature of the particular polmer employed, the rate of drug release can be controlled. Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues. The injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable media just prior to use.

The therapeutic compositions of the present invention can include pharmaceutically acceptable salts of the components therein, *e.g.*, which may be derived from inorganic or organic acids. By "pharmaceutically acceptable salt" is meant those salts which are, within the scope of sound medical judgement, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well-known in the art. For example, S. M. Berge, et al. describe pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences* (1977) 66:1 *et seq.*, which is incorporated

herein by reference. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like. The salts may be prepared *in situ* during the final isolation and purification of the compounds of the invention or separately by reacting a free base function with a suitable organic acid. Representative acid addition salts include, but are not limited to acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsufonate, digluconate, glycerophosphate, hemisulfate, heptonoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxymethanesulfonate (isethionate), lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartate, thiocyanate, phosphate, glutamate, bicarbonate, p-toluenesulfonate and undecanoate. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl, and diamyl sulfates; long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides; arylalkyl halides like benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained. Examples of acids which may be employed to form pharmaceutically acceptable acid addition salts include such inorganic acids as hydrochloric acid, hydrobromic acid, sulphuric acid and phosphoric acid and such organic acids as oxalic acid, maleic acid, succinic acid and citric acid.

As used herein, the terms "pharmaceutically acceptable," "physiologically tolerable" and grammatical variations thereof as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal with a minimum of undesirable

physiological effects such as nausea, dizziness, gastric upset and the like. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipeints which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The restin polypeptides of the present invention can also be included in a composition comprising a prodrug. As used herein, the term "prodrug" refers to compounds which are rapidly transformed *in vivo* to yield the parent compound, for example, by enzymatic hydrolysis in blood. A thorough discussion is provided in T. Higuchi and V. Stella, *Prodrugs as Novel Delivery Systems*, Vol. 14 of the ACS Symposium Series and in Edward B. Roche, ed., *Bioreversible Carriers in Drug Design*, American Pharmaceutical Association and Permagon Press, 1987, both of which are incorporated herein by reference. As used herein, the term "pharmaceutically acceptable prodrug" refers to (1) those prodrugs of the compounds of the present invention which are, within the scope of sound medical judgement, suitable for use in contact with the tissues of humans and animals without undue toxicity, irritation, allergic response and the like, commensurate with a suitable benefit-to-risk ratio and effective for their intended use and (2) zwitterionic forms, where possible, of the parent compound.

The dosage of the restin of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound.

For treating humans or animals, about 10 mg/kg of body weight to about 20 mg/kg of body weight of the restin protein or the apomigren protein can be administered. In combination therapies, *e.g.*, the restin protein or apomigren protein of the invention in combination with radiotherapy, chemotherapy, or immunotherapy, it may be possible to reduce the dosage, *e.g.*, to about 0.1 mg/kg of body weight to about 0.2 mg/kg of body weight. Depending upon the half-life of the restin in the particular animal or human, the restin can be administered between several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time. In addition, restin and/or apomigren can be administered in conjunction with other forms of therapy, *e.g.*, chemotherapy, radiotherapy, or immunotherapy.

The restin formulations include those suitable for oral, rectal, ophthalmic (including intravitreal or intracameral), nasal, topical (including buccal and sublingual), intrauterine, vaginal or parenteral (including subcutaneous, intraperitoneal, intramuscular, intravenous, intradermal, intracranial, intratracheal, and epidural) administration. The restin formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only

the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

When a therapeutically effective amount of protein of the present invention is administered orally, the restin protein of the present invention will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% protein of the present invention, and preferably from about 25 to 90% protein of the present invention. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of protein of the present invention, and preferably from about 1 to 50% protein of the present invention.

When a therapeutically effective amount of protein of the present invention is administered by intravenous, cutaneous or subcutaneous injection, protein of the present invention will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to protein of the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The amount of protein of the present invention in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of protein of the present invention with which to treat each individual patient. Initially, the attending physician will administer low doses of protein of the present invention and observe the patient's response. Larger doses of protein of the present invention may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the protein of the present invention will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question. Optionally, cytotoxic agents may be incorporated or otherwise combined with restin proteins, or biologically functional protein fragements thereof, to provide dual therapy to the patient.

The therapeutic compositions are also presently valuable for veterinary applications. Particularly domestic animals and thoroughbred horses, in addition to humans, are desired patients for such treatment with proteins of the present invention.

Cytotoxic agents such as ricin, are linked to restin, and high affinity restin protein fragments, thereby providing a tool for destruction of cells that bind restin.

These cells may be found in many locations, including but not limited to, micrometastases and primary tumors. Proteins linked to cytotoxic agents are infused in a manner designed to maximize delivery to the desired location. For example, ricin-linked high affinity restin fragments are delivered through a cannula into vessels supplying the target site or directly into the target. Such agents are also delivered in a controlled manner through osmotic pumps coupled to infusion cannulae. A combination of restin antagonists may be co-applied with stimulators of angiogenesis to increase vascularization of tissue. This therapeutic regimen provides an effective means of destroying metastatic cancer.

Additional treatment methods include administration of restin, restin fragments, restin analogs, restin antisera, or restin receptor agonists and antagonists linked to cytotoxic agents. It is to be understood that the restin can be human or animal in origin. Restin can also be produced synthetically by chemical reaction or by recombinant techniques in conjunction with expression systems. Restin can also be produced by enzymatically cleaving isolated plasminogen or plasmin to generate proteins having anti-angiogenic activity. Restin may also be produced by compounds that mimic the action of endogenous enzymes that cleave plasminogen to restin. Restin production may also be modulated by compounds that affect the activity of plasminogen cleaving enzymes.

These protein sequences are compared to known sequences using protein sequence databases such as GenBank, Brookhaven Protein, SWISS-PROT, and PIR to determine potential sequence homologies. This information facilitates elimination of sequences that exhibit a high degree of sequence homology to other molecules, thereby enhancing the potential for high specificity in the development of antisera, agonists and antagonists to restin.

The present invention also encompasses gene therapy whereby a polynucleotide encoding restin or apomigren, or a mutant, fragment, or fusion protein thereof, is introduced and regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as gene therapy, are disclosed in *Gene Transfer into Mammalian Somatic Cells in vivo*, N. Yang (1992) *Crit. Rev. Biotechn.* 12(4):335-

356, which is hereby incorporated by reference. Gene therapy encompasses incorporation of DNA sequences into somatic cells or germ line cells for use in either ex vivo or in vivo therapy. Gene therapy functions to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

Strategies for treating these medical problems with gene therapy include therapeutic strategies such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene for the product protein that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a gene such as restin may be placed in a patient and thus prevent occurrence of angiogenesis; or a gene that makes tumor cells more susceptible to radiation could be inserted and then radiation of the tumor would cause increased killing of the tumor cells.

Many protocols for transfer of restin DNA or restin regulatory sequences are envisioned in this invention. Transfection of promoter sequences, other than one normally found specifically associated with restin, or other sequences which would increase production of restin protein are also envisioned as methods of gene therapy. An example of this technology is found in Transkaryotic Therapies, Inc., of Cambridge, Mass., using homologous recombination to insert a "genetic switch" that turns on an erythropoietin gene in cells. See *Genetic Engineering News*, Apr. 15, 1994. Such "genetic switches" could be used to activate restin (or the restin receptor) in cells not normally expressing restin (or the restin receptor) .

Gene transfer methods for gene therapy fall into three broad categories: physical (*e.g.*, electroporation, direct gene transfer and particle bombardment), chemical (*e.g.*, lipid-based carriers, or other non-viral vectors) and biological (*e.g.*, virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA

to macrophages and Kupffer cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer, and *in vitro* gene transfer. In *ex vivo* gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, the transfected cells are expanded in number and then reimplanted in the patient. In *in vitro* gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, the transfected cells are selected and expanded for either implantation into a patient or for other uses.

In vivo gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. Methods include using virally mediated gene transfer using a noninfectious virus to deliver the gene in the patient or injecting naked DNA into a site in the patient and the DNA is taken up by a percentage of cells in which the gene product protein is expressed. Additionally, the other methods described herein, such as use of a "gene gun," may be used for in vitro insertion of restin DNA or restin regulatory sequences.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to transfer the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, make a complex that can cross the cell membrane and provide the DNA into the interior of the cell. Another chemical method uses receptor-based endocytosis, which involves binding a specific ligand to a cell surface receptor and enveloping and transporting it across the cell membrane. The ligand binds to the DNA and the whole complex is transported into the cell. The ligand gene complex is injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, or other somatic cells. These altered cells are then introduced into the patient to provide the gene product from the inserted DNA.

Viral vectors have also been used to insert genes into cells using in vivo protocols. To direct the tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue-specific can be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus infected surrounding cells which also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors that have been used for gene therapy protocols include but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors. Murine leukemia retroviruses are composed of a single strand RNA complexed with a nuclear core protein and polymerase (pol) enzymes, encased by a protein core (gag) and surrounded by a glycoprotein envelope (env) that determines host range. The genomic structure of retroviruses include the *gag*, *pol*, and *env* genes enclosed at by the 5' and 3' long terminal repeats (LTR). Retroviral vector systems exploit the fact that a minimal vector containing the 5' and 3' LTRs and the packaging signal are sufficient to allow vector packaging, infection and integration into target cells providing that the viral structural proteins are supplied in trans in the packaging cell line. Fundamental advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types,

precise single copy vector integration into target cell chromosomal DNA, and ease of manipulation of the retroviral genome.

The adenovirus is composed of linear, double stranded DNA complexed with core proteins and surrounded with capsid proteins. Advances in molecular virology have led to the ability to exploit the biology of these organisms to create vectors capable of transducing novel genetic sequences into target cells *in vivo*. Adenoviral-based vectors will express gene product proteins at high levels. Adenoviral vectors have high efficiencies of infectivity, even with low titers of virus. Additionally, the virus is fully infective as a cell free virion so injection of producer cell lines are not necessary. Another potential advantage to adenoviral vectors is the ability to achieve long term expression of heterologous genes *in vivo*.

Mechanical methods of DNA delivery include fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion, lipid particles of DNA incorporating cationic lipid such as lipofectin, polylysine-mediated transfer of DNA, direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun," and inorganic chemical approaches such as calcium phosphate transfection. Particle-mediated gene transfer methods were first used in transforming plant tissue. With a particle bombardment device, or "gene gun," a motive force is generated to accelerate DNA-coated high density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Particle bombardment can be used in *in vitro* systems, or with *ex vivo* or *in vivo* techniques to introduce DNA into cells, tissues or organs. Another method, ligand-mediated gene therapy, involves complexing the DNA with specific ligands to form ligand-DNA conjugates, to direct the DNA to a specific cell or tissue.

It has been found that injecting plasmid DNA into muscle cells yields high percentage of the cells which are transfected and have sustained expression of marker genes. The DNA of the plasmid may or may not integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative

tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent, transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. This technique can be used in *in vitro* systems, or with *ex vivo* or *in vivo* techniques to introduce DNA into cells, tissues or organs.

Carrier mediated gene transfer *in vivo* can be used to transfect foreign DNA into cells. The carrier-DNA complex can be conveniently introduced into body fluids or the bloodstream and then site-specifically directed to the target organ or tissue in the body. Both liposomes and polycations, such as polylysine, lipofectins or cytofectins, can be used. Liposomes can be developed which are cell specific or organ specific and thus the foreign DNA carried by the liposome will be taken up by target cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoportein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

The transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then resides in the cytoplasm or in the nucleoplasm. DNA can be coupled to carrier nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

Gene regulation of restin may be accomplished by administering compounds that bind to the restin gene, or control regions associated with the restin gene, or its

corresponding RNA transcript to modify the rate of transcription or translation. Additionally, cells transfected with a DNA sequence encoding restin may be administered to a patient to provide an *in vivo* source of restin. For example, cells may be transfected with a vector containing a nucleic acid sequence encoding restin. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue, or may be non-patient cells.

For example, tumor cells removed from a patient can be transfected with a vector capable of expressing the restin protein of the present invention, and re-introduced into the patient. The transfected tumor cells produce restin levels in the patient that inhibit the growth of the tumor. Patients may be human or non-human animals. Cells may also be transfected by non-vector, or physical or chemical methods known in the art such as electroporation, ionoporation, or via a "gene gun." Additionally, restin DNA may be directly injected, without the aid of a carrier, into a patient. In particular, restin DNA may be injected into skin, muscle or blood.

The gene therapy protocol for transfecting restin into a patient may either be through integration of the restin DNA into the genome of the cells, into minichromosomes or as a separate replicating or non-replicating DNA construct in the cytoplasm or nucleoplasm of the cell. Restin expression may continue for a long-period of time or may be reinjected periodically to maintain a desired level of the restin protein in the cell, the tissue or organ or a determined blood level.

In addition, the invention encompasses antibodies and antisera, which can be used for testing of novel anti-angiogenic proteins, and can also be used in diagnosis, prognosis, or treatment of diseases and conditions characterized by, or associated with, angiogenic activity or lack thereof. Such antibodies and antisera can also be used to up-regulate angiogenesis where desired, *e.g.*, in post-infarct heart tissue, antibodies or antisera to the restin protein can be used to block localized, native anti-angiogenic proteins and processes, and increase formation of new blood vessels and inhibit atrophy of heart tissue. In addition, restin and apomigren can be used to induce apoptosis, or antibodies thereof can be used to prevent apoptosis in a cell or tissue. Assays for apoptosis are provided in U.S.S.N. XX/XXX,XXX, "Anti-Angiogenic Peptides and Methods of Use Thereof," by Vikas P. Sukhatme, filed

December 8, 1998, and in Dhanabal *et al.* (1998) (Endostatin Induces Endothelial Cell Apoptosis," *J. Biol. Chem.*, submitted).

Such antibodies and antisera can be combined with pharmaceutically-acceptable compositions and carriers to form diagnostic, prognostic or therapeutic compositions. The term "antibody" or "antibody molecule" refers to a population of immunoglobulin molecules and/or immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antibody combining site or paratope.

Passive antibody therapy using antibodies that specifically bind restin can be employed to modulate angiogenic-dependent processes such as reproduction, development, and wound healing and tissue repair. In addition, antisera directed to the Fab regions of restin antibodies can be administered to block the ability of endogenous restin antisera to bind restin.

The restin of the present invention also can be used to generate antibodies that are specific for the inhibitor and its receptor. The antibodies can be either polyclonal antibodies or monoclonal antibodies. These antibodies that specifically bind to the restin or restin receptors can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify the restin or restin receptors in a body fluid or tissue. Results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer and other angiogenic mediated diseases.

The invention also includes use of restin, antibodies to restin, and compositions comprising restin and/or its antibodies in diagnosis or prognosis of diseases characterized by angiogenic activity. As used herein, the term "prognostic method" means a method that enables a prediction regarding the progression of a disease of a human or animal diagnosed with the disease, in particular, an angiogenesis dependent disease. The term "diagnostic method" as used herein means a method that enables a determination of the presence or type of angiogenesis-dependent disease in or on a human or animal.

The restin can be used in a diagnostic method and kit to detect and quantify antibodies capable of binding restin. These kits would permit detection of

circulating restin antibodies which indicates the spread of micrometastases in the presence of restin secreted by primary tumors in situ. Patients that have such circulating anti-restin antibodies may be more likely to develop multiple tumors and cancers, and may be more likely to have recurrences of cancer after treatments or periods of remission. The Fab fragments of these anti-restin antibodies may be used as antigens to generate anti-restin Fab-fragment antisera which can be used to neutralize anti-restin antibodies. Such a method would reduce the removal of circulating restin by anti-restin antibodies, thereby effectively elevating circulating restin levels.

The present invention also includes isolation of receptors specific for restin. Protein fragments that possess high affinity binding to tissues can be used to isolate the restin receptor on affinity columns. Isolation and purification of the restin receptor is a fundamental step towards elucidating the mechanism of action of restin. Isolation of an restin receptor and identification of restin agonists and antagonists will facilitate development of drugs to modulate the activity of the restin receptor, the final pathway to biological activity. Isolation of the receptor enables the construction of nucleotide probes to monitor the location and synthesis of the receptor, using *in situ* and solution hybridization technology. Further, the gene for the restin receptor can be isolated, incorporated into an expression vector and transfected into cells, such as patient tumor cells to increase the ability of a cell type, tissue or tumor to bind restin and inhibit local angiogenesis.

Restin proteins are employed to develop affinity columns for isolation of the restin receptor from cultured tumor cells. Isolation and purification of the restin receptor is followed by amino acid sequencing. Using this information the gene or genes coding for the restin receptor can be identified and isolated. Next, cloned nucleic acid sequences are developed for insertion into vectors capable of expressing the receptor. These techniques are well known to those skilled in the art. Transfection of the nucleic acid sequence(s) coding for restin receptor into tumor cells, and expression of the receptor by the transfected tumor cells enhances the responsiveness of these cells to endogenous or exogenous restin and thereby decreasing the rate of metastatic growth.

Angiogenesis-inhibiting proteins of the present invention can be synthesized in a standard microchemical facility and purity checked with HPLC and mass spectrophotometry. Methods of protein synthesis, HPLC purification and mass spectrophotometry are commonly known to those skilled in these arts. Restin proteins and restin receptors proteins are also produced in recombinant *E. coli* or yeast expression systems, and purified with column chromatography.

Different protein fragments of the intact restin molecule can be synthesized for use in several applications including, but not limited to the following; as antigens for the development of specific antisera, as agonists and antagonists active at restin binding sites, as proteins to be linked to, or used in combination with, cytotoxic agents for targeted killing of cells that bind restin. The amino acid sequences that comprise these proteins are selected on the basis of their position on the exterior regions of the molecule and are accessible for binding to antisera. The amino and carboxyl termini of restin, as well as the mid-region of the molecule are represented separately among the fragments to be synthesized.

The synthetic protein fragments of restin have a variety of uses. The protein that binds to the restin receptor with high specificity and avidity is radiolabeled and employed for visualization and quantitation of binding sites using autoradiographic and membrane binding techniques. This application provides important diagnostic and research tools. Knowledge of the binding properties of the restin receptor facilitates investigation of the transduction mechanisms linked to the receptor.

Restin and restin-derived proteins can be coupled to other molecules using standard methods. The amino and carboxyl termini of restin both contain tyrosine and lysine residues and are isotopically and nonisotopically labeled with many techniques, for example radiolabeling using conventional techniques (tyrosine residues-chloramine T, iodogen, lactoperoxidase; lysine residues-Bolton-Hunter reagent). These coupling techniques are well known to those skilled in the art. Alternatively, tyrosine or lysine is added to fragments that do not have these residues to facilitate labeling of reactive amino and hydroxyl groups on the protein. The coupling technique is chosen on the basis of the functional groups available on the amino acids including, but not limited to amino, sulfhydral, carboxyl, amide, phenol,

and imidazole. Various reagents used to effect these couplings include among others, glutaraldehyde, diazotized benzidine, carbodiimide, and p-benzoquinone.

Restin proteins are chemically coupled to isotopes, enzymes, carrier proteins, cytotoxic agents, fluorescent molecules, chemiluminescent, bioluminescent and other compounds for a variety of applications. The efficiency of the coupling reaction is determined using different techniques appropriate for the specific reaction. For example, radiolabeling of an restin protein with ^{125}I is accomplished using chloramine T and $Na^{125}I$ of high specific activity. The reaction is terminated with sodium metabisulfite and the mixture is desalted on disposable columns. The labeled protein is eluted from the column and fractions are collected. Aliquots are removed from each fraction and radioactivity measured in a gamma counter. In this manner, the unreacted $Na^{125}I$ is separated from the labeled restin protein. The protein fractions with the highest specific radioactivity are stored for subsequent use such as analysis of the ability to bind to restin antisera.

In addition, labeling restin proteins with short lived isotopes enables visualization of receptor binding sites *in vivo* using positron emission tomography or other modern radiographic techniques to locate tumors with restin binding sites.

Systematic substitution of amino acids within these synthesized proteins yields high affinity protein agonists and antagonists to the restin receptor that enhance or diminish restin binding to its receptor. Such agonists are used to suppress the growth of micrometastases, thereby limiting the spread of cancer. Antagonists to restin are applied in situations of inadequate vascularization, to block the inhibitory effects of restin and promote angiogenesis. For example, this treatment may have therapeutic effects to promote wound healing in diabetics.

The restin protein of the present invention can also be used as a nutritional source or supplement. Such uses include without limitation use as a protein or amino acid supplement, use as a carbon source, use as a nitrogen source and use as a source of carbohydrate. In such cases, the restin protein of the invention can be added to the food of a particular organism, or can be administered as a separate solid or liquid preparation, such as in the form of powder, pills, solutions, suspensions or capsules.

In the case of microorganisms, the protein or polynucleotide of the invention can be added to the medium in or on which the microorganism is cultured.

The invention is further illustrated by the following examples, which are not meant to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof, which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLES

Example 1: Cells and cell lines

C-PAE (cow pulmonary artery endothelial, ATCC No. CCL-209) cells, obtained from ATCC (American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, 20110-2209, USA) were maintained in DMEM medium with 10% fetal calf serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM L-glutamine. The cells were incubated in a humidified environment at 37°C in the presence of 5% CO_2. IMR-90, lung fibroblast and IC-21, macrophage precursor cells were purchased from ATCC and maintained under similar conditions as the C-PAE cells. HMVE-L cells (human microvascular endothelial cells of lung origin, Clonetics, San Diego, California, USA) were maintained in EGM-2MV medium.

Example 2: Isolation of restin

The following CDNA libraries were used as template for DNA-PCR.

K562 - erthyroid library

JMN - human mesothiolioma

HFK - human fetal kidney library

786-0 - human renal cell carcinoma

MAB - human adult brain

293 - control

Primers corresponding to C-terminal portion of the Collagen XV NC10 domain were synthesized from GIBCO-BRL (Life Technologies, Gaithersburg, Maryland, USA). The primers used were 5'-TTT TTT GAA TTC ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT TTG-3' (upstream primer) and 5'-AAG AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TCG AT-3' (downstream primer).

Amplification was carried out using standard protocols. Briefly, 100 ng of DNA from each cDNA library was used as template. Amplification was carried out for 30 cycles with the following parameters: 94°C for denaturation, 60°C for annealing, and 72°C for extension, each for 1 minute.

The 540 bp amplified fragment, designated "restin" herein, was purified and digested with *Eco*RI and *Not*I. Cloning of Collagen XV into the *Pichia* expression vector (pPICZαA, InVitrogen, San Diego, California, USA) is shown schematically Fig. 5. The vector pPICZαA was also digested with the above restriction enzymes and ligated with the restin fragment. Initial transformation was carried out with the host strain Top 10F' (InVitrogen, San Diego, California, USA). Positive clones, designated "pPICZαA-CXV," were sequenced to confirm the modification. The plasmid was then linearized with *Sac*I enzyme and used for homologous recombination into the yeast host strain GS115 (InVitrogen, San Diego, California, USA) via lithium chloride transformation. The recombination was carried out as described in the *Pichia* expression manual. The colonies were allowed to grow for two days at 30°C on YPD/Zeocin plates. Clones which grew on Zeocin were selected for small scale induction. The induction procedure was carried out as suggested by the manufacturer (InVitrogen, San Diego, California, USA). Restin was successfully derived from the K592 and HFK libraries. The HFK clone was used for all subsequence investigations.

Example 3: Expression of Restin in Pichia expression system

A single colony was inoculated from YPD/Zeocin into 25 ml of BMGY/Zeo (100 ug/ml) in a 500 ml baffled flask. The culture was grown at 30°C in a shaking

incubator (250 rpm) until the culture reached an OD600 2-6 (approximately 16-24 hours). The cells in the log phase of growth were used in a 1:100 dilution to inoculate a 1-liter culture. 500 ml of BMGY medium was added, and the cells were grown in a 2-liter baffled flask and the culture reached an OD600 15-20 (2-3 days). The cells were harvested by centrifugation at 5000 rpm for 10 minutes at room temperature. The supernatant was decanted, and the cell pellet resuspended in 300 - 400 ml of BMMY medium to induce expression of the recombinant protein. To maintain induction, 100% methanol was added to a final concentration of 0.5% every 24 hours. Supernatant was collected on the second, third, and fourth days after induction, and analyzed for protein expression by Coomassie stained SDS-PAGE.

Example 4: Purification of Restin using Heparin-Sepharose column

The supernatant was concentrated by precipitation with 60-70% ammonium sulfate. The precipitated protein was centrifuged at 10,000 rpm for 10 min and the pellet was resuspended in phosphate-buffered saline and dialyzed overnight at 4°C. The final dialysis was done with 10 mM Tris and 50 mM NaCl, pH 7.4. A polyprep column was packed with 5 ml of Heparin-Sepharose resin (Pharmacia Biotech, Inc., Piscataway, New Jersey, USA) and equilibrated with 10 mM Tris, 50 MM NaCl, pH 7.4. The concentrated crude protein sample was loaded in 20-30 ml batches onto the column at a flow rate of 10-15 ml/hour. The column was washed with equilibration buffer until the absorbance at 280 nm was greater than 0.001. The elution of bound proteins was carried by step-wise gradients of NaCl (0.2M, 0.6M and 1.0 M). The elution profile failed to show any distinct protein peak, and when the samples were analyzed by SDS-PAGE, a majority of the protein did not bind to the column. Lowering the sodium chloride concentration and changing the pH did not change the binding profile. The recombinant protein did not bind to the heparin column.

xample 5: Construction of His.Tag Restin recombinant protein

A His.Tag motif to the restin molecule at the amino terminus to simplify purification. Two complementary oligonucleotide primers were synthesized, 5'-AAT TCC ATC ACC ATC ACC ATC ACG-3' (upstream), and 5'-AAT TCG TGA TGG TGA TGG TGA TGG-3' (downstream). These primers code for histidine residues (six histidine residue at a stretch), flanked by the *Eco*RI restriction site. The construct pPICZαA-CXV was digested with *Eco*RI. Fifty µl of each primer (100 µM) was mixed and denatured at 95°C for 5 minutes and cooled immediately on ice. The annealed primer was ligated into *Eco*RI digested vector backbone at 16°C overnight. The ligase was heat-inactivated at 70°C for 10 minutes and the excess primer was removed using Glass Max purification column (Gibco/BRL, Gaithersburg, Maryland, USA). The ligated vector was transformed using the host strain Top 10F' (InVitrogen, San Diego, California, USA). The recombinant clones were screened by PCR and restriction enzyme analysis. The presence of His.Tag motif was further confirmed by sequencing, and the construct was designated "pPICZαA/His.Restin."

The recombinant pPICZαA/His.Restin was linearized with *Sac*I and recombination was carried out using the strain GS115 (InVitrogen, San Diego, California, USA).

Example 6: Homology of Apomigren with C-terminus of mouse and human endostatin

GenBank was searched for sequences that showed homology to endostatin. One such sequence was the C-terminal region of human type XV collagen. Fig. 9 shows the 85 C-terminal amino acids of the full length α1 chain of collagen XV in relation to the entire chain. Fig. 10 shows the homology between the 85 extreme C-terminus amino acids of human collagen XV and human and mouse endostatin. This region of human collagen XV has been designated herein as apomigren. About 60% amino acid identity exists between the C-terminal amino acids of these proteins. When conservative substitutions are accounted for, these regions share about 75% amino acid identity.

Example 7: Cloning, expression and purification of human apomigren

The sequence encoding the C-terminal 85 amino acids (255 bp) of human collagen XV (Myers *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10144-48) was amplified using Vent DNA polymerase, on the pPICzαA-CXV vector as template, which contains the partial NC10 domain (555 bp). Amplification was performed for 30 cycles with denaturation at 94°C, annealing at 60°C and extension at 72°C, each for 1 minute, with the following primers: 5'-TTC CAT ATG ATA TAC TCC TTT GAT GGT CGA GAC ATA ATG ACA-3' and 5'-AAG AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TC GAT-3' The amplified DNA (255 bp) was purified using a QIAquick purification kit (Qiagen, Hilden, Germany) digested with *Nde*I and *Not*I (these sites are underlined in the primer), and ligated into a *Nde*I and *Not*I predigested pET28(a) expression vector (Novagen, Madison, Wisconsin, USA) so as to give an expected protein sequence of MGSSHHHHHHSSGLVPAGSHM-Apomigren, which is designated herein as "apomigren". (Fig. 10). A histidine tag was used from the expression vector to assist in the purification procedures, since there was no guarantee that apomigren would bind heparin, as does endostatin. The expression, processing and purification of the recombinant apomigren was performed using a Ni-NTA column in the presence of 8 M urea as described by Dhanabal *et al.* (1999) ("Cloning, Expression and *in vitro* Activity of Human Endostatin," *Cancer Research*, in press), and also in U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-Angiogenic Proteins," by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of all of which are herein incorporated by reference. A two-step pH protocol (pH8.0 and 2.0) was used to elute the protein from the column, and the results are shown in Fig. 11, which is a graph showing the elution pH (left y-axis) and absorbance at 280 nM (right y-axis) for each fraction (x-axis). A small peak was observed around fraction 4, and a large peak at fraction 23. Samples of 15 ml were taken from selected fractions were analyzed by SDS-PAGE, and the results are shown in Fig. 12. Lane 1 contains size marker, lane 2, apomigren protein lysate prior to loading on the Ni-NTA column; lane 2, flowthrough from the column; lane 4, fraction 4; lanes 5 and 6, fraction 23,

non-reduced and reduced, respectively; lane 7, fraction 24; lane 8, fraction 27. There was no protein in the flow-through fraction (comparison of lane 3 with lane 2), suggesting that apomigren bound tightly to the Ni-NTA column. Some amount of protein eluted in the pH 8.0 wash (lane 4, fraction 4), but most of the protein eluted in the pH 2.0 strip solution (lanes 5-8). Higher molecular weight complexes are seen corresponding to 23, 35, 46, and 69 kDa. Lanes 5 and 6 are non-reduced and reduced samples of fraction 23 and a single discrete band of 14 kDa was observed in the reduced sample. In reducing conditions with DTT, all the higher complexes are converted to a single band of 14 kDa in size. The majority of the protein was found in fractions 20-26.

Fractions containing purified apomigren (fractions 20-26) were pooled and refolded slowly with step-wise dialysis in decreasing urea concentrations. The final dialysis was carried out against sterile 1X PBS, pH 7.4 at 4°C. During dialysis, although some protein precipitated out of solution, a significant fraction remained in soluble form. The soluble protein was stored at -70°C in small aliquots and a fresh aliquot was used for each assay. The concentration of the protein was determined by BCA protein assay (Pierce Chemical Co., Rockford, Illinois, USA). Endotoxin levels were determined on the purified soluble protein by performing the LAL assay (Associates of Cape Cod, Inc., Falmouth, Massachusetts, USA) and the levels were about 6 units/ml, with a protein concentration of 200 µg/ml. In previous experiments, these levels had no visible effects on any of the assays used. Western blot analysis was performed on the purified protein with a histidine tag antibody and showed positive immunoreactivity.

Example 8: Apomigren alters the morphology of endothelial cells

When apomigren protein (0.5 - 1.0 µg/ml) was added to endothelial cells, a dramatic change occurred in the morphology of these cells. The cells appeared rounded, detached, and membrane blebbing, cytoplasmic shrinkage, and chromatin condensation was observed, as is typically seen in apoptotic cells. These changes are shown in Fig. 13, which is a set of three photomicrographs. Confluent C-PAE cells were trypsinized, plated at approximately 50% confluency in 2% FBS

containing 3 ng/ml bFGF with simultaneous addition of apomigren at 0.05 µg/ml and 0.7 µg/ml. Pictures were taken 24 hours after treatment (400X magnification). Increasing concentrations of apomigren showed increasing number of cells with apoptotic morphology. Figs. 13B and 13C show the apomigren treated cells (0.05 mg/ml and 0.07 mg/ml, respectively) in comparison with untreated control endothelial cells (Fig. 13A). Such changes were not observed in non-endothelial cells such as IMR-90.

The apoptotic morphology was also seen in endothelial same cells upon treatment with mouse endostatin, but only at 5-10-fold higher concentrations. Human endostatin had no visible effect upon these cells.

Example 9: Proliferation of endothelial cells

To test the anti-proliferative effects of apomigren on C-PAE cells, the thymidine incorporation assay was performed as described in Dhanabal *et al.* (1999) ("Cloning, Expression and *in vitro* Activity of Human Endostatin," *Cancer Research*, in press), and in U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-angiogenic Proteins", by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of which are incorporated herein by reference.. Based on earlier data with endostatin, initial experiments were done with concentrations of apomigren in the 1-20 µg/ml range. C-PAE cells were plated in 24-well plates coated with fibronectin (10 mg/ml) coated plates at 12,500 cells per well in 0.5 ml DMEM containing 2% FBS. After a 24-hour incubation at 37°C, the medium was replaced with fresh DMEM and 2% FBS containing 3 ng/ml of bFGF (R & D systems, Minneapolis, Minnesota, USA) with or without the protein being tested. The cells were pulsed with 1 mCi of ^{3}H-thymidine for 24 hours. Medium was aspirated, cells were washed three times with PBS, and then solubilized by addition of 1.5 N NaOH (100 ml per well) and incubated at 37°C for 30 minutes. Cell-associated radioactivity was determined with a liquid scintillation counter.

In the present test, the inhibition of methyl ^{3}H-thymidine uptake in response to bFGF (3 ng/ml and 2% FBS) and purified soluble apomigren protein expressed

from bacteria was tested in C-PAE cells. The results are graphed in Fig. 14. Concentration of apomigren (●), and mouse (□) and human (○) endostatin are shown on the x-axis, and ^{3}H-thymidine incorporation is on the y-axis. Each data point is a mean of triplicate values from a representative experiment. The DNA synthesis in the control samples (with bFGF alone) were considered as 100%. Comparison between apomigren and mouse and human endostatin at certain concentrations are indicated. In general, a dose-dependent inhibition of bFGF-induced proliferation was seen in C-PAE cells. At 1 μg/ml of apomigren, the proliferation of C-PAE cells was completely inhibited, to below that of basal activity (*i.e.*, no bFGF). Lower concentrations of apomigren, *e.g.*, 0.05-0.9 μg/ml, showed a dose dependent effect, 0.1 μg/ml with an ED_{50} value in the range of 300-400 ng/ml. When compared with data generated previously for endostatin, the activity of apomigren was clearly superior.

Example 10: Cell cycle assay

C-PAE and IMR-90 cells were growth arrested by contact inhibition for 48 hours in complete medium. The cells were harvested by trypsinization and seeded into a 6 well plate coated with fibronectin (10 μg/ml). Each well was seeded with 0.2 to 0.3 x 10^6cells in 1% FCS supplemented with 3 ng/ml of bFGF. For the dose response study, different concentrations of human apomigren were added and the cells harvested at 21-24 hours after treatment. The cells were washed in PBS buffer and fixed in 70% ice cold ethanol. The fixed cells were rehydrated at room temperature for 30 minutes in PBS buffer containing 2% FCS and 0.1% Tween-20. The cells were then centrifuged at 1500x g for 10 minutes and resuspended in 0.5 ml of the above PBS buffer to which RNase (5 μg/ml) was added. RNase digestion was carried at 37°C for 1 hour, followed by staining with propidium iodide (5 μg/ml). The cells were analyzed using a Becton Dickinson FACStar plus flow cytometer (Becton Dickinson, Waltham, Massachusetts, USA). For calculating the percentage of cells in different phases of the cell cycle, the ModFit software was used. The protocol was generally performed as described by Dhanabal *et al.* (1999) ("Cloning,

Expression and *in vitro* Activity of Human Endostatin," *Cancer Research*, in press), and in U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-angiogenic Proteins", by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of which are incorporated herein by reference.

The results are shown in Fig. 15, which is a bar graph. The x-axis shows concentration of apomigren, ranging from 0 to 1000 ng/ml, and the y-axis shows percentage of cells in S-phase. C-PAE cells are represented by dark bars and IMR-90 cells by white bars. Control cells treated with bFGF and 1% FBS alone were considered as 100%. The contact-inhibited cells showed 7% cells in S-phase. After trypsinization, plating out at about 50% confluence, and on stimulation with bFGF (3 ng/ml) and 1% FBS, a 4.8-fold increase in S-phase cells was observed after 21 hours (Fig. 15). A dose-dependent G1 cell cycle arrest was seen with apomigren concentrations ranging from 0.06-1.0 μg/ml, with an ED_{50} of approximately 500 ng/ml. To check the specificity of this effect, apomigren was also tested on IMR-90 fibroblast cells. For IMR-90 cells, complete medium (10% FBS) was used as the stimulus. A 14-fold increase in S-phase cells was observed when compared to the contact-inhibited cells, however, in the presence of apomigren, no cell cycle arrest was seen at those same doses which showed an effect in endothelial cells. In experiments using complete medium as the stimulus for endothelial cells, apomigren caused cell cycle arrest in a dose-dependent fashion just as seen with bFGF stimulation, suggesting that apomigren can counter the stimulus of several growth factors.

Example 11: Apoptosis of endothelial cells and Annexin V - FITC assay

Since apomigren inhibits proliferation of endothelial cells and showed dramatic morphological effects on these cells, apomigren was studied for its ability to induce apoptosis in endothelial cells. After initiation of apoptosis, most cell types translocate the membrane phospholipid phosphatidylserine (PS) from the inner surface of the plasma membrane to the outside (van Engeland *et al.* (1998) Cytometry 31:1-9; Zhang *et al.* (1997) Biotechniques 23:525-31; Koopman *et al.*

(1994) Blood 84:1415-20). PS, and therefore apoptosis, can be detected by staining with an FITC conjugate of Annexin V, a calcium-dependent phospholipid binding protein of 38 kDa protein that has a high affinity for PS. PS can generally be detected prior to bleb formation and DNA fragmentation.

Annexin V, a calcium dependent phospholipid binding protein with a high affinity for phosphtidylserine (PS) was used to detect early stage apoptosis. Briefly, 200,000 cells were plated onto a fibronectin coated 6-well plate in DMEM containing 2% FBS and 3 ng/ml of bFGF. Different concentrations of recombinant apomigren were added to each well, and cells were harvested and processed 18 hours after treatment. Cells were stained with propidium iodide (PI) just before analysis. Gating was performed to analyze only Annexin V positive and PI negative cells. Human recombinant TNF-α (40 ng/ml) was used as a positive control. The remainder of the protocol was carried out as described by Dhanabal *et al.* (1999) ("Cloning, Expression and *in vitro* Activity of Human Endostatin," *Cancer Research*, in press), and in U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-angiogenic Proteins", by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of which are incorporated herein by reference.

When proliferating C-PAE cells were treated with apomigren at different concentrations ranging from 0.1-1 μg/ml, increasing numbers of cells were rounded, and they detached from the fibronectin-coated plate. In the controls, the cells showed intact endothelial cell morphology, whereas the cells treated with apomigren appeared rounded and showed membrane blebbing characteristics of apoptotic cells. The mean fluorescence intensity difference between control and apomigren treated cells was significant ($p = 0.01$) at 0.05-1.0 μg/ml. This experiment was repeated with HMVE-L cells, and apomigren concentrations in the range of 2.5-5 ug/ml showed comparable shift in fluorescence as noted for the positive controls TNF-α (40 ng/ml) and 5% ethanol. Intracellular caspase activity was also measured in apomigren treated C-PAE cells, and showed a 2-fold increase when compared with control cells.

Example 12: Endothelial cell migration assay

The migration assay with ECV304 and IC-21 cells was carried out as described by Dhanabal *et al.* (1999) ("Cloning, Expression and *in vitro* Activity of Human Endostatin," *Cancer Research*, in press), and in U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-angiogenic Proteins", by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of which are herein incorporated by reference. Generally, the migration assay was performed using 12-well Boyden chemotaxis chambers (Neuro-Probe, Inc., Cabin John, Maryland, USA) with a polycarbonate membrane (25 x 80 mm PVD free, 8 μ pores, Poretics Corp., Livermore, California, USA). The non-specific binding of growth factor to the chambers was prevented by coating the chambers with a solution containing 0.5% gelatin, 1 mM $CaCl_2$ and 150 mM NaCl at 37°C overnight. ECV304 cells were grown in 10% FBS containing 5 ng/ml DiI (1,1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate DiIC18, Molecular Probes, Eugene, Oregon, USA) overnight and washed with PBS containing 0.5% BSA. Following trypsinization, the cells were counted using Coulter-Counter Z1, (Luton, U.K.) and diluted to 300,000 cells/ml in Medium 199 (Gibco/BRL, Gaithersburg, Maryland, USA) containing 0.5% FBS. The lower chamber was filled with Medium 199 containing 25 ng/ml bFGF. The upper chamber was seeded with 15,000 cells/well with different concentrations of recombinant protein. Cells were allowed to migrate for 4 hours at 37°C. At that time, the cells on the upper surface of the membrane were removed with a cell scraper and the (migrated) cells on the lower surface were fixed in 3% formaldehyde and washed in PBS. Images of the fixed membrane were obtained using fluorescence microscopy at 550 nM with a digital camera and the number of cells on each membrane was determined using the OPTIMAS (version 6.0) software (Media Cybernetics, L.P., Silver Spring, MD, USA).

Since C-PAE cells do not migrate in response to bFGF and VEGF, human ECV304 cells were used for this assay with different concentrations of apomigren and bFGF as stimulus. The results are shown in Fig. 16, which is a graph showing concentration of various proteins on the x-axis and percent cells that migrated in

response to bFGF on the y-axis. The control is apomigren-treated IC-21 cells (■), and treatments consist of apomigren (●) and mouse (□) and human (○) endostatin on ECV304 cells.

Apomigren had no effect on the migration of IC-21 macrophage precursor cells at these doses, but dramatic inhibition of migration was observed in endothelial cells at doses of 0.15-1.25 μg/ml, with 150 ng/ml resulting in about 80% inhibition. (Fig. 16). Previous work showed that endostatin inhibits migration of ECV304endothelial cells, but at higher doses of (ED_{50} ~5 ng/ml and 80% inhibition at 10-15 μg/ml) (Dhanabal *et al.* (1999) "Cloning, Expression and *in vitro* Activity of Human Endostatin," *Cancer Research*, in press).

The effects of restin on cell migration were also studied. The assay was conducted as for apomigren, and the results are shown in Figure 17. The effect of restin on inhibition of renal cancer cell (RCC) tumor growth were also studied, and were conducted as described in U.S.S.N. XX/XXX,XXX, "Anti-Angiogenic Peptides and Methods of Use Thereof," by Vikas P. Sukhatme, filed December 8, 1998, and U.S.S.N. XX/XXX,XXX, "Methods of Producing Anti-Angiogenic Proteins," by Vikas P. Sukhatme, filed December 8, 1998, the entire teachings of all of which are herein incorporated by reference.

The results are shown in Figure 18, which shows that restin (●) performed favorably relative to endostatin (○) in inhibiting tumor growth, relative to untreated control tumor (■).

Example 13: Effect of Angiostatin in a Renal Tumor Model

Athymic nude mice were transplanted subcutaneously with renal tumor cell line 786-0. Approximately 5 million cells in 200 l of phosphate buffered saline were inoculated in the flank of each mouse. Each tumor was allowed to reach approximately 200 cubic millimeters.

About 20 micrograms of purified recombinant restin or endostatin (1 mg/kg) was given to each animal intraperitoneally on a daily basis for 3 weeks. The results are shown in Fig. 18, which show that restin performed favorably against endostatin.

Example 14: CAM Assay

The ability of apomigren to block bFGF induced angiogenesis *in vivo* was tested using the chorioallantoic membrane (CAM) assay. Fertilized white Leghorn chicken eggs (SPAFAS, Inc., Norwich CT) were opened on 100 mm^2 petri dishes and allowed to grow until day 11 in a humidified incubator at 38%C. Pellets containing vitrogen (Collagen Biomaterials, Palo Alto, CA) at a concentration of 0.73 mg/ml and supplemented with vehicle alone, VEGF + FGF-2, or his.apomigren (0.5, 1, 5, 10, 20, or 50 μg/ml) were allowed to polymerize at 37°C for 2 hours. The pellets were placed on a nylon mesh and oriented on the periphery of the CAM. Embryos were returned to the incubator for 24 hours. Invasion of new capillaries on the collagen mesh was assessed by injection of FITC-dextran into the circulation of the chicken embryo. At the end of the experiment, the meshes were dissected and evaluation of vascular density was done using the program NIH Image 1.59. Assays were performed in triplicate and four independent experiments were conducted. The results are shown in Fig. 19.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. Isolated restin, comprising an amino acid sequence of about 170 to about 200 amino acid residues, and having at least 70% sequence identity with the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen, wherein the isolated restin is characterized as having anti-angiogenic activity.

2. Isolated restin, comprising the amino acid sequence of SEQ ID NO: , wherein the isolated restin is characterized as having anti-angiogenic activity.

3. An isolated polynucleotide encoding an anti-angiogenic protein, the protein comprising 170 to 200 amino acid residues, and further comprising the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen.

4. An isolated polynucleotide encoding an anti-angiogenic protein comprising the amino acid sequence of SEQ ID NO: .

5. The isolated polynucleotide of Claim 3, comprising (a) the nucleotide sequence of SEQ ID NO: , (b) a sequence complementary to the nucleotide sequence of SEQ ID NO: , or (c) a sequence that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO: .

6. An isolated polynucleotide, comprising the nucleotide sequence amplified by the primers of 5'-TTT TTT GAA TTC ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT TTG-3' and 5'-AAG AAT GCG GCC GCT TAC TTC CTA GCG TCT GTC ATG AAA CTG TTT TCG AT-3'.

7. An anti-angiogenic fragment of the isolated restin of Claim 2, comprising at least 25 contiguous amino acids of the amino acid sequence of SEQ ID NO:

8. An anti-angiogenic fragment of isolated restin, the fragment comprising 80 to 90 contiguous amino acids corresponding to the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen.

9. An anti-angiogenic fragment of the isolated restin of Claim 2, the fragment comprising amino acid 97 to amino acid 181 of SEQ ID NO: .

10. An isolated polynucleotide encoding an anti-angiogenic protein comprising a fragment of the amino acid sequence of SEQ ID NO: , the fragment comprising the amino acid sequence comprising amino acid 97 to amino acid 181 of SEQ ID NO: .

11. An isolated polynucleotide comprising (a) a fragment of the nucleotide sequence of SEQ ID NO: , the fragment comprising the nucleotide sequence comprising nucleotide 289 to nucleotide 543 of SEQ ID NO: , (b) a sequence complementary a fragment of the nucleotide sequence of SEQ ID NO: , the fragment comprising the nucleotide sequence comprising nucleotide 289 to nucleotide 543 of SEQ ID NO: , or (c) a sequence that hybridizes under stringent conditions to a fragment of the nucleotide sequence of SEQ ID NO: , the fragment comprising the nucleotide sequence comprising nucleotide 289 to nucleotide 543 of SEQ ID NO: .

12. A polynucleotide which is an allelic variant of the isolated polynucleotide of Claim 3.

13. An isolated polynucleotide of Claim 11, wherein the polynucleotide is operably linked to an expression control sequence.

14. A host cell transformed with the polynucleotide of Claim 13.

15. The host cell of Claim 14, where the cell is selected from the group comprising bacterial, yeast, mammalian, insect or plant cells.

16. A process for producing a protein encoded by the polynucleotide of Claim 11, wherein the process comprises:

(a) growing a culture of a host cell transformed with the polynucleotide of Claim 11, where the host cell is selected from the group comprising bacterial, yeast, mammalian, insect or plant cells; and

(b) purifying the protein from the culture;

thereby producing the protein encoded by the polynucleotide of Claim 11.

17. A fusion protein, comprising the isolated restin of Claim 1 and one or more additional proteins.

18. The fusion protein of Claim 17, further comprising at least one protein molecule selected from the group comprising: restin, endostatin, angiostatin, apomigren, or endostatin mutant fragment EM 1.

19. A fusion protein, comprising the isolated restin fragment of Claim 7 and one or more additional proteins.

20. The fusion protein of Claim 19, further comprising at least one protein molecule selected from the group comprising: restin, endostatin, angiostatin, apomigren, or endostatin mutant fragment EM 1.

21. A fusion protein, comprising the isolated restin fragment of Claim 9 and one or more additional proteins.

22. The fusion protein of Claim 21, further comprising at least one protein molecule selected from the group comprising: restin, endostatin, angiostatin, apomigren, or endostatin mutant fragment EM 1.

23. A composition comprising, as a biologically active ingredient, the isolated restin of Claim 1.

24. The composition of Claim 23, and a pharmaceutically-compatible carrier.

25. A composition comprising, as a biologically active ingredient, one or more isolated restin fragments of Claim 7.

26. The composition of Claim 25, and a pharmaceutically-compatible carrier.

27. A composition comprising, as a biologically active ingredient, the isolated restin fragment of Claim 9.

28. The composition of Claim 27, and a pharmaceutically-compatible carrier.

29. A composition comprising, as a biologically active ingredient, the fusion protein of Claim 17.

30. A composition comprising, as a biologically active ingredient, the fusion protein of Claim 19.

31. A composition comprising, as a biologically active ingredient, the fusion protein of Claim 21.

32. A method for inhibiting angiogenic activity in mammalian tissue, the method comprising contacting the tissue with a composition comprising the amino acid sequence of SEQ ID NO: .

33. The method of Claim 32, wherein the composition comprises an amino acid sequence comprising the amino acid 97 to amino acid 181 of SEQ ID NO: .

34. A method of using the composition of Claim 23 to inhibit a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

35. The method of Claim 34, wherein the disease is selected from the group comprising angiogenesis-dependent cancers, benign tumors, rheumatoid arthritis, psoriasis, ocular angiogenesis diseases, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, wound granulation, intestinal adhesions, atherosclerosis, scleroderma, hypertrophic scars, cat scratch disease, *Heliobacter pylori* ulcers, dialysis graft vascular access stenosis, contraception, and obesity.

36. The method of Claim 35, wherein the disease is cancer.

37. The method of Claim 36, wherein the disease is renal cancer.

38. A method of using the composition of Claim 23 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

39. The method of Claim 38, wherein the disease is cancer.

40. The method of Claim 39, wherein the disease is renal cancer.

41. A method of using the composition of Claim 24 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

42. The method of Claim 41, wherein the disease is cancer.

43. The method of Claim 42, wherein the disease is renal cancer.

44. A method of using the composition of Claim 25 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

45. The method of Claim 44, wherein the disease is cancer.

46. The method of Claim 45, wherein the disease is renal cancer.

47. A method of using the composition of Claim 26 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

48. The method of Claim 47, wherein the disease is cancer.

49. The method of Claim 48, wherein the disease is renal cancer.

50. A method of using the composition of Claim 27 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

51. The method of Claim 50, wherein the disease is cancer.

52. The method of Claim 51, wherein the disease is renal cancer.

53. A method of using the composition of Claim 28 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

54. The method of Claim 53, wherein the disease is cancer.

55. The method of Claim 54, wherein the disease is renal cancer.

56. A method of using the composition of Claim 29 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

57. The method of Claim 56, wherein the disease is cancer.

58. The method of Claim 57, wherein the disease is renal cancer.

59. A method of using the composition of Claim 30 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

60. The method of Claim 59, wherein the disease is cancer.

61. The method of Claim 60, wherein the disease is renal cancer.

62. A method of using the composition of Claim 31 to treat a disease characterized by angiogenic activity, the method comprising administration of the composition to a patient with the disease.

63. The method of Claim 62, wherein the disease is cancer.

64. The method of Claim 63, wherein the disease is renal cancer.

65. A process for providing a mammal with restin protein, the process comprising introducing mammalian cells into a human, said mammalian cells having been treated *in vitro* to insert therein the polynucleotide of SEQ ID NO: and expressing *in vivo* in said mammal a therapeutically effective amount of the restin protein.

66. The process of Claim 65, wherein the cells are lymphocytes.

67. The process of Claim 66, wherein the lymphocytes are chosen from the group comprising T-lymphocytes and B-lymphocytes.

68. The process of Claim 65, wherein the cells are chosen from the group comprising: blood cells, TIL cells, bone marrow cells, vascular cells, tumor cells, liver cells, muscle cells, fibroblast cells.

69. The process of claim 65, wherein the polynucleotide is inserted into the cells by a viral vector.

70. A process for providing a mammal with apomigren protein, the process comprising introducing mammalian cells into a human, said mammalian cells having been treated *in vitro* to insert therein a polynucleotide comprising nucleotide 289 to nucleotide 543 of SEQ ID NO: and expressing *in vivo* in said mammal a therapeutically effective amount of the apomigren protein.

71. The process of Claim 70, wherein the cells are lymphocytes.

72. The process of Claim 71, wherein the lymphocytes are chosen from the group comprising T-lymphocytes and B-lymphocytes.

73. The process of Claim 70, wherein the cells are chosen from the group comprising: blood cells, TIL cells, bone marrow cells, vascular cells, tumor cells, liver cells, muscle cells, fibroblast cells.

74. The process of claim 60, wherein the polynucleotide is inserted into the cells by a viral vector.

75. A process for producing an isolated polynucleotide, the process comprising the steps of:

(a) preparing one or more polynucleotide probes that hybridize under conditions of moderate stringency to a nucleotide sequence selected from the group consisting of:

(i) SEQ ID NO: ;

(ii) an isolated polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: ; and

(iii) an isolated polynucleotide encoding 181 contiguous amino acids corresponding to the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen;

(b) hybridizing said probe(s) to mammalian DNA; and

(c) isolating the DNA polynucleotide detected with the probe(s);

wherein the nucleotide sequence of the isolated polynucleotide corresponds to the nucleotide sequence of SEQ ID NO: .

76. An isolated polynucleotide produced according to the process of Claim 75.

77. An isolated polynucleotide comprising the polynucleotide of Claim 76.

78. A process for producing an isolated polynucleotide, the process comprising the steps of:

(a) preparing one or more polynucleotide primers that hybridize under conditions of moderate stringency to a nucleotide sequence selected from the group consisting of:

(i) SEQ ID NO: ;

(ii) an isolated polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: ; and

(iii) an isolated polynucleotide encoding 181 contiguous amino acids corresponding to the C-terminus of the NC10 domain of the α1 chain of human Type XV collagen;

(b) hybridizing said primer(s) to mammalian DNA;

(c) amplifying mammalian DNA sequences; and

(d) isolating the polynucleotide product of step (c),

wherein the nucleotide sequence of said isolated polynucleotide corresponds to the nucleotide sequence of SEQ ID NO: .

79. An isolated polynucleotide produced according to the process of Claim 78.

80. An isolated polynucleotide comprising the polynucleotide of Claim 79.

81. Antibodies to the isolated restin of Claim 1.

82. Antibodies to the isolated restin of Claim 2.

83. Antibodies to the fragments of isolated restin of Claim 7.

84. Antibodies to the fragments of isolated restin of Claim 8.

85. Antibodies to the fragments of isolated restin of Claim 9.

86. An isolated mutant, derivative, analog or homolog of the restin protein of Claim 1.

87. An isolated polynucleotide that encodes the restin mutant, derivative, analog or homolog of Claim 86.

Sequence Range: 1-546
Upstream primers for Restin and Apomigren are underlined, downstream primer for both is double underlined. Primer nucleotides not in the Restin sequence are shown in lower case.

ttt ttt gaa ttc->
5 10 15 20 25 30 35 40 45
->ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT TTG GCT GCT CTG
TAA AGT TCA CGG TTA ATA CTC TTC GGA CGA GAC GTA AAC CGA CGA GAC

50 55 60 65 70 75 80 85 90 95
AAC ATG CCA TTT TCT GGG GAC ATT CGA GCT GAT TTT CAG TGC TTC AAG
TTG TAC GGT AAA AGA CCC CTG TAA GCT CGA CTA AAA GTC ACG AAG TTC

100 105 110 115 120 125 130 135 140
CAG GCC AGA GCT GCA GGA CTG TTG TCC ACC TAC CGA GCA TTC TTA TCT
GTC CGG TCT CGA CGT CCT GAC AAC AGG TGG ATG GCT CGT AAG AAT AGA

145 150 155 160 165 170 175 180 185 190
TCC CAT TTG CAA GAT CTG TCC ACC ATT GTG AGG AAA GCA GAG AGA TAC
AGG GTA AAC GTT CTA GAC AGG TGG TAA CAC TCC TTT CGT CTC TCT ATG

195 200 205 210 215 220 225 230 235 240
AGC CTT CCC ATA GTG AAC CTC AAG GGC CAA GTA CTT TTT AAT AAT TGG
TCG GAA GGG TAT CAC TTG GAG TTC CCG GTT CAT GAA AAA TTA TTA ACC

245 250 255 260 265 270 275 280 285
GAC TCA ATT TTT TCT GGC CAC GGA GGT CAG TTC AAT ATG CAT ATT CCA
CTG AGT TAA AAA AGA CCG GTG CCT CCA GTC AAG TTA TAC GTA TAA GGT

ttt cca tat g->
290 295 300 305 310 315 320 325 330 335
->ATA TAC TCC TTT GAT GGT CGA GAC ATA ATG ACA GAT CCT TCT TGG CCC
TAT ATG AGG AAA CTA CCA GCT CTG TAT TAC TGT CTA GGA AGA ACC GGG

340 345 350 355 360 365 370 375 380
CAG AAA GTC ATT TGG CAT GGC TCC AGC CCC CAT GGC GTC CGC CTT GTG
GTC TTT CAG TAA ACC GTA CCG AGG TCG GGG GTA CCG CAG GCG GAA CAC

385 390 395 400 405 410 415 420 425 430
GAT AAC TAC TGT GAA GCA TGG CGA ACC GCG GAC ACA GCG GTC ACG GGA
CTA TTG ATG ACA CTT CGT ACC GCT TGG CGC CTG TGT CGC CAG TGC CCT

435 440 445 450 455 460 465 470 475 480
CTT GCC TCC CCG CTG AGC ACG GGG AAG ATT CTG GAC CAG AAA GCA TAC
GAA CGG AGG GGC GAC TCG TGC CCC TTC TAA GAC CTG GTC TTT CGT ATG

485 490 495 500 505 510 515 520 525
AGC TGT GCT AAT CGG CTA ATT GTC CTA TGT ATC GAA AAC AGT TTC ATG
TCG ACA CGA TTA GCC GAT TAA CAG GAT ACA TAG CTT TTG TCA AAG TAC

530 535 540 545
ACA GAC GCT AGG AAG TAA
TGT CTG CGA TCC TTC ATT cgc cgg cgt aag aa

Fig. 1

Sequence Range: 1 to 182

```
     5      10     15      20      25     30      35      40     45
  ISS ANY EKP ALH LAA LNM PFS GDI RAD FQC FKQ ARA AGL LST YRA FLS

  50      55     60      65      70     75      80      85     90      95
  SHL QDL STI VRK AER YSL PIV NLK GQV LFN NWD SIF SGH GGQ FNM HIP

    100    105     110     115    120     125     130    135     140
  IYS FDG RDI MTD PSW PQK VIW HGS SPH GVR LVD NYC EAW RTA DTA VTG

145    150     155     160    165     170     175    180
  LAS PLS TGK ILD QKA YSC ANR LIV LCI ENS FMT DAR K
```

Fig. 2

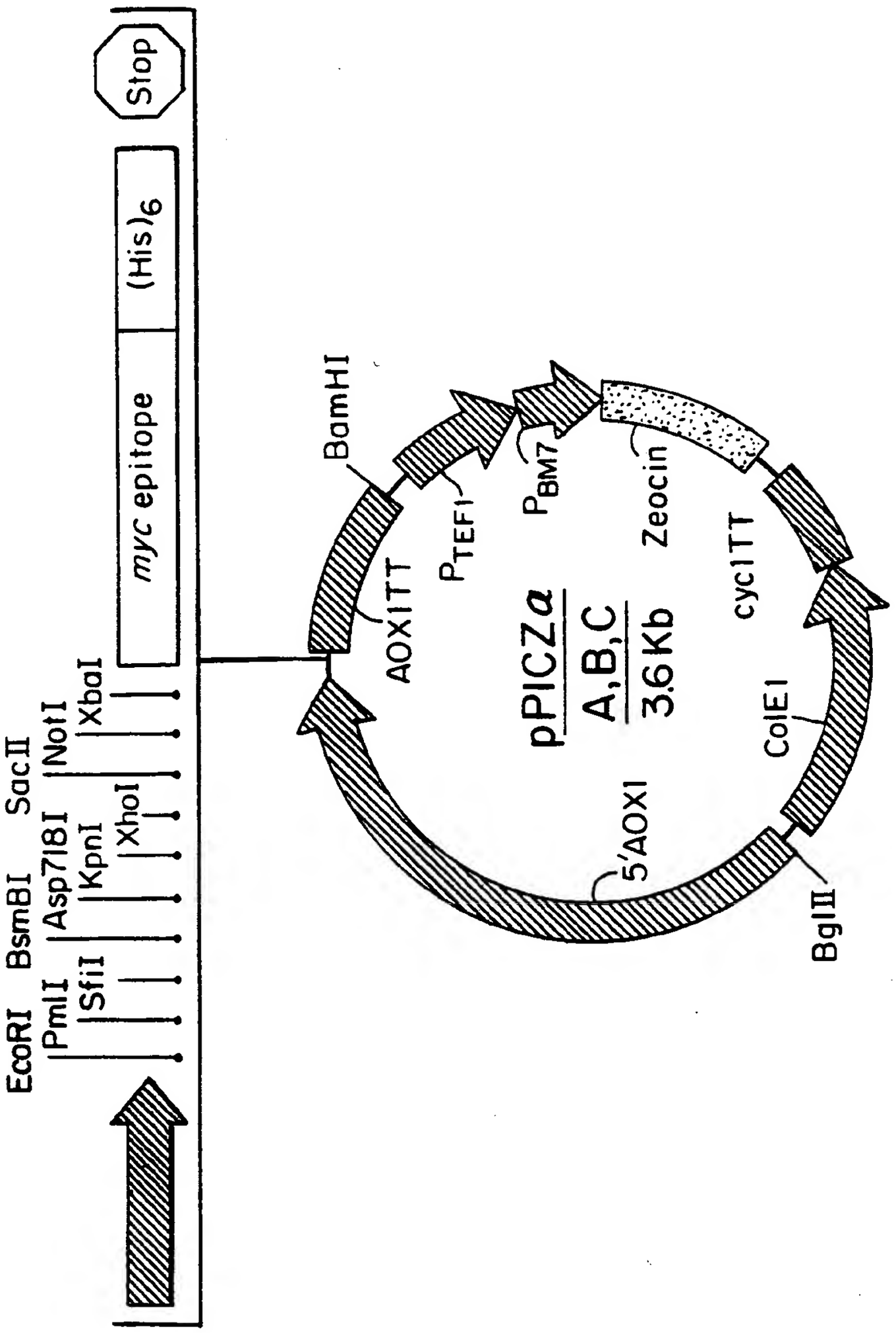
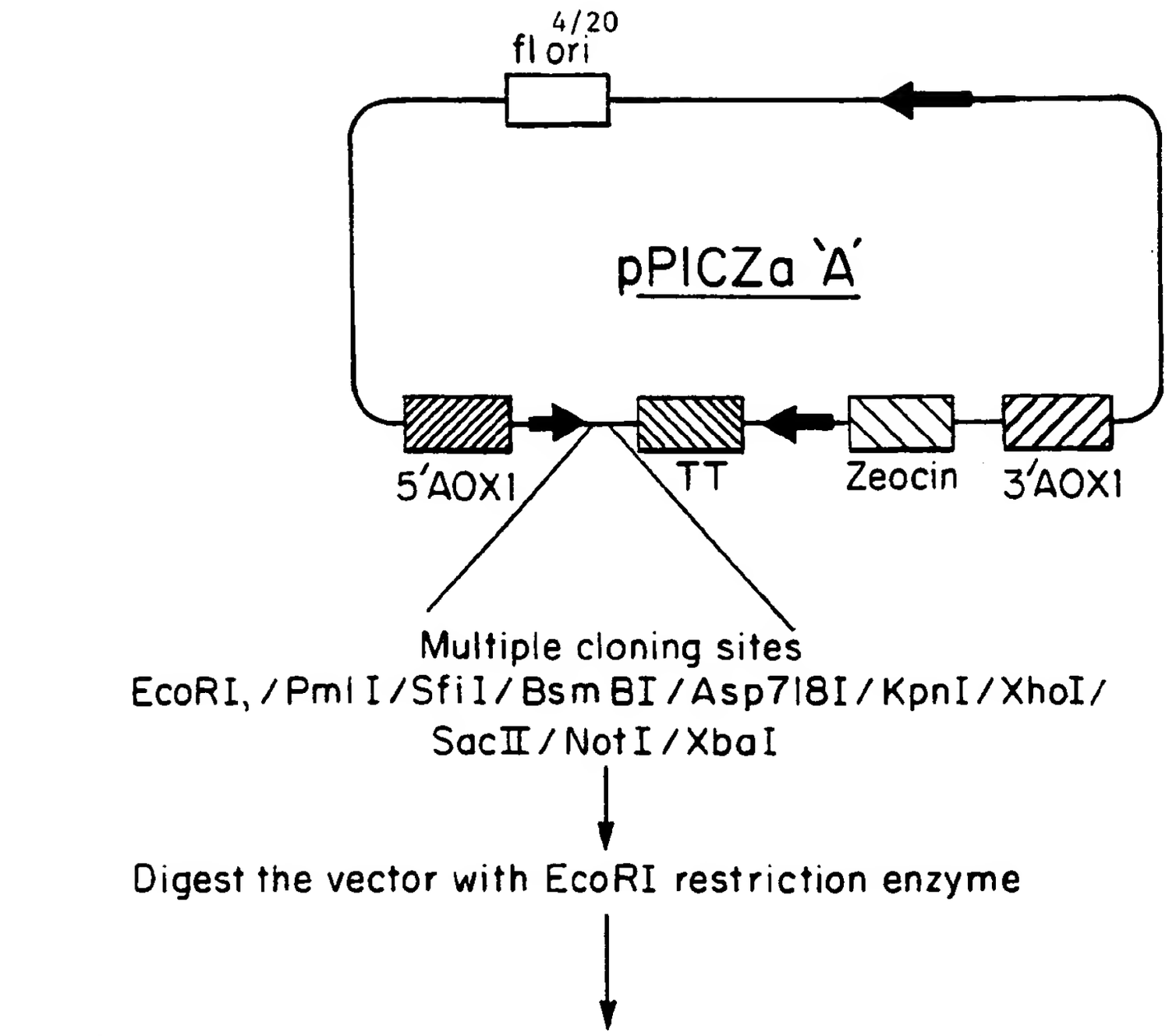

EcoRI
PmlI
BsmBI
Asp718I
KpnI
SacII
SfiI
NotI
XhoI
XbaI
myc epitope
(His)6
Stop
BamHI
AOX1TT
PTEF1
PBM7
pPICZα
A,B,C
3.6Kb
Zeocin
cyc1TT
ColE1
5'AOX1
BglII

Fig. 3


fl ori
pPICZa 'A'
5'AOX1
TT
Zeocin
3'AOX1
Multiple cloning sites
EcoRI, / PmlI / SfiI / BsmBI / Asp718I / KpnI / XhoI /
SacII / NotI / XbaI
Digest the vector with EcoRI restriction enzyme
Denature and anneal two complimentary oligo which would incorporate His.Tag motif and NdeI, NheI restriction sites (His. Tag region would be helpful to simplify purification and the presence of NdeI and NheI restriction sites facilitate shuttling of PCR from prokaryotic expression system to yeast expression without going through PCR amplification process)
5' AAT TCC ATC ACC ATC ACC ATC ACC ATA TGG CTA GCA 3'
5' AAT TTG CTA GCC ATA TGG TGA TGG TGA TGG TGA TGG 3'

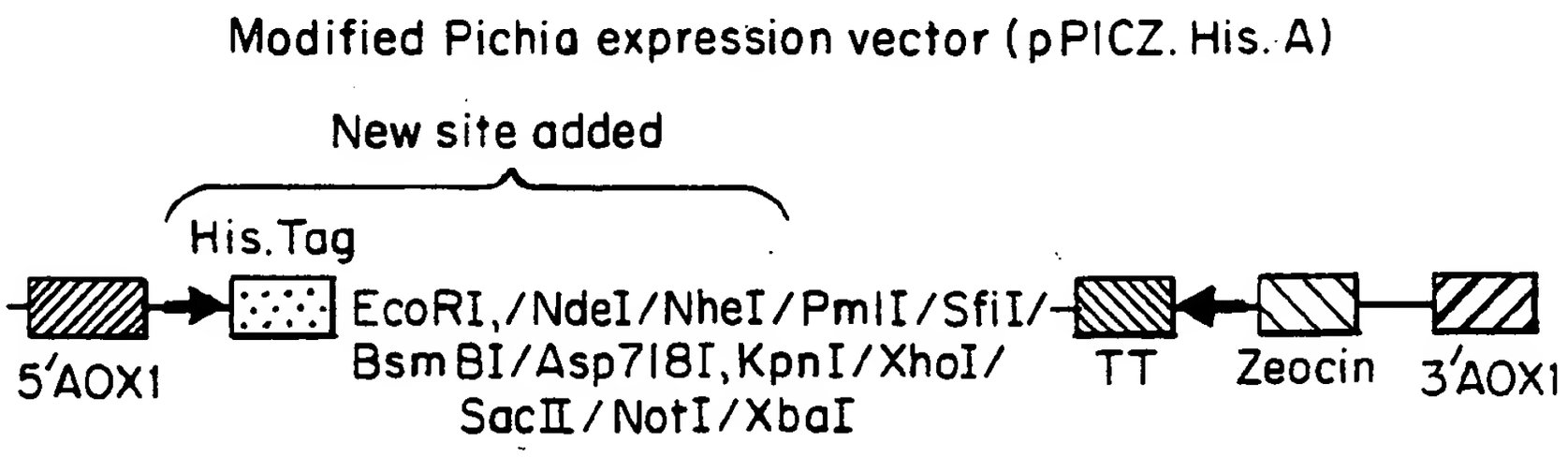

Modified Pichia expression vector (pPICZ. His. A)
New site added
His.Tag
5'AOX1
EcoRI, / NdeI / NheI / PmlI / SfiI /
BsmBI / Asp718I, KpnI / XhoI /
SacII / NotI / XbaI
TT
Zeocin
3'AOX1

Fig. 4

Flow Chart: Cloning of Restin (ColXV) into Pichia Expression System

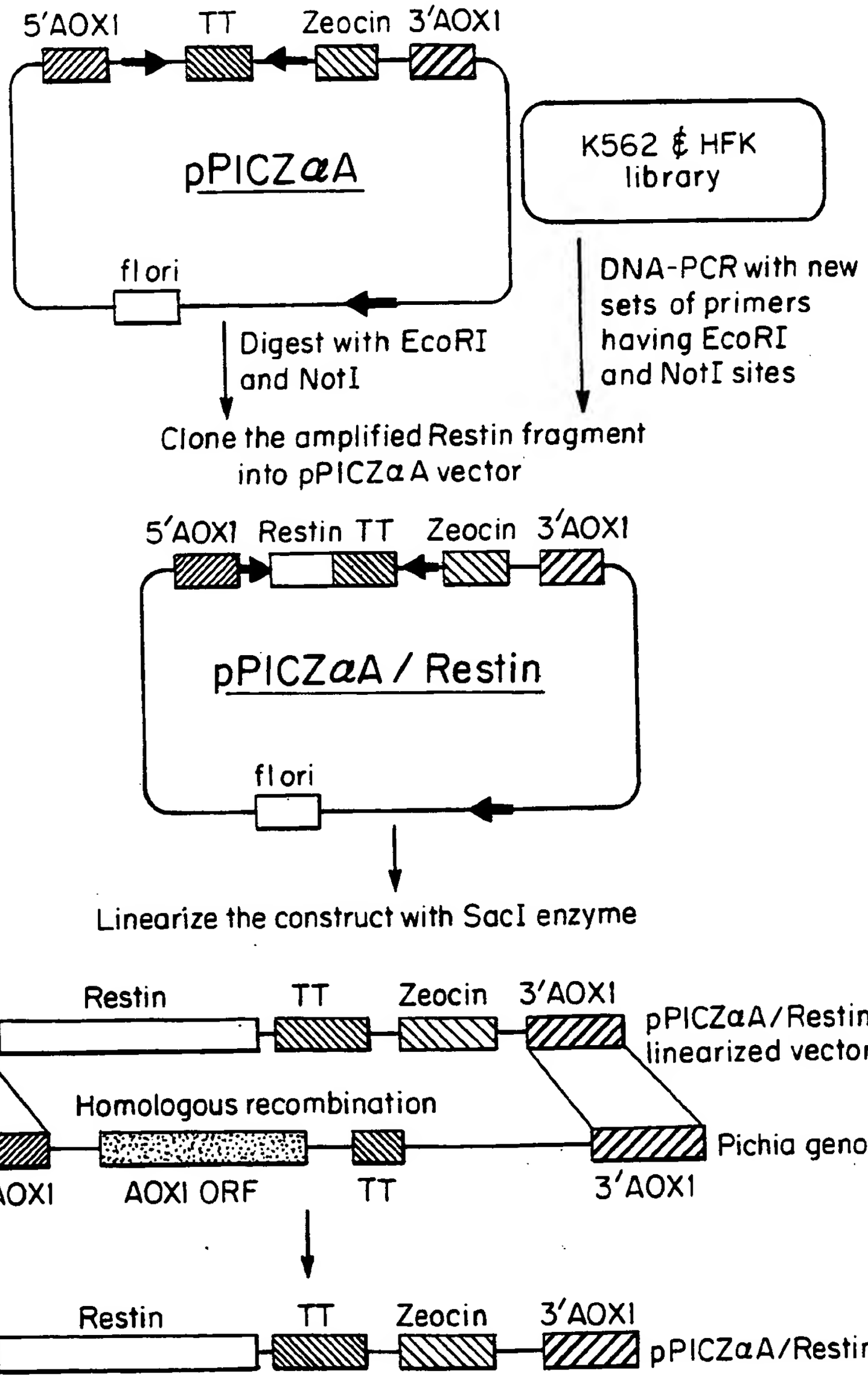


Fig. 5

FIG. 6

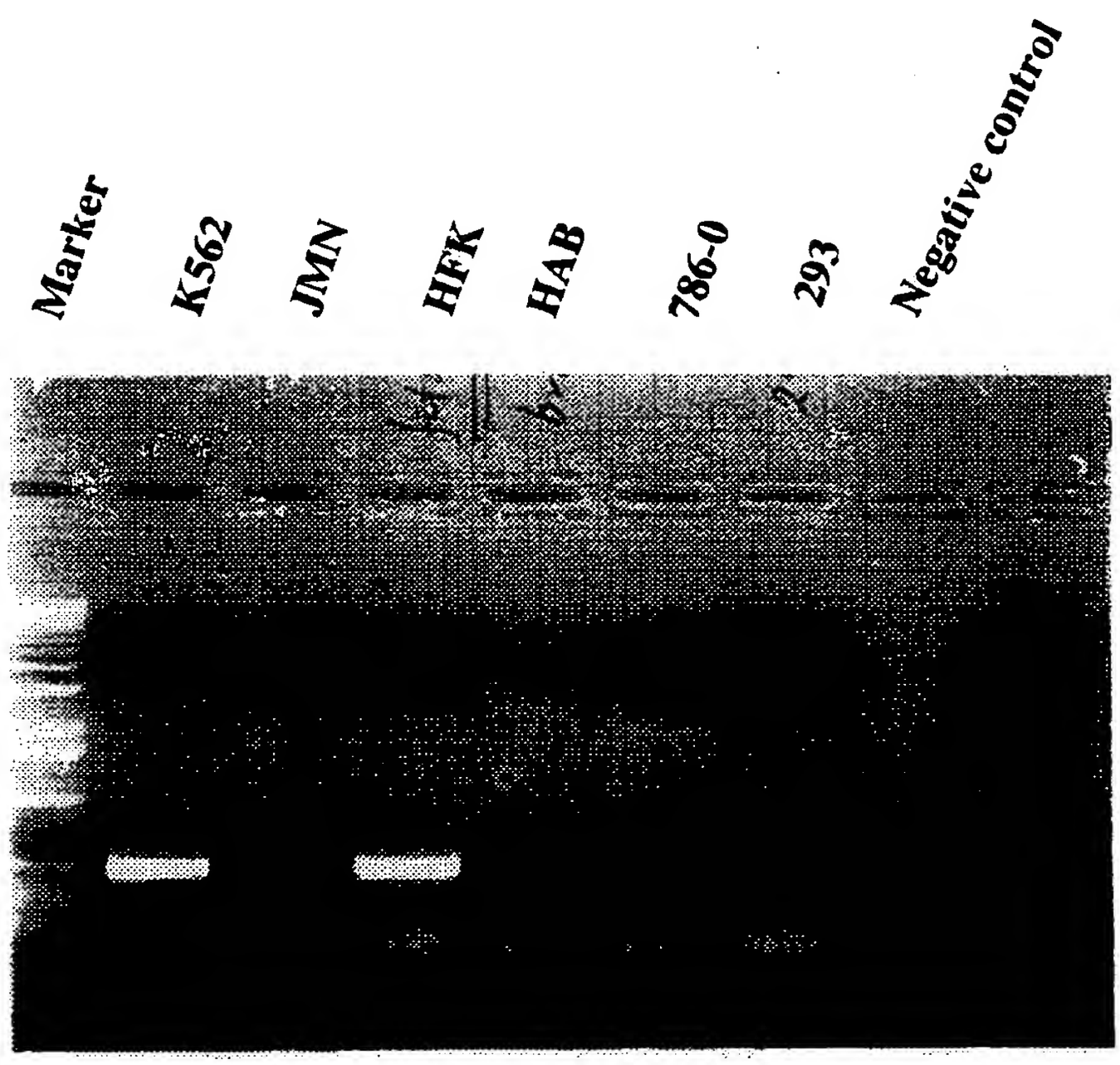


Fig. 7A

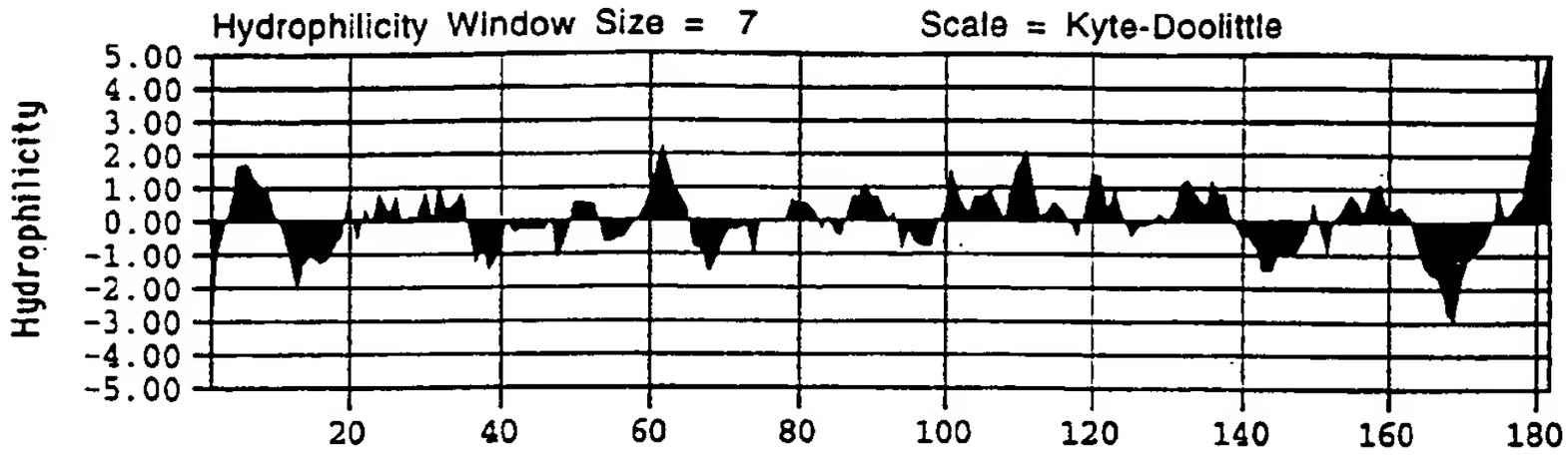


Fig. 7B

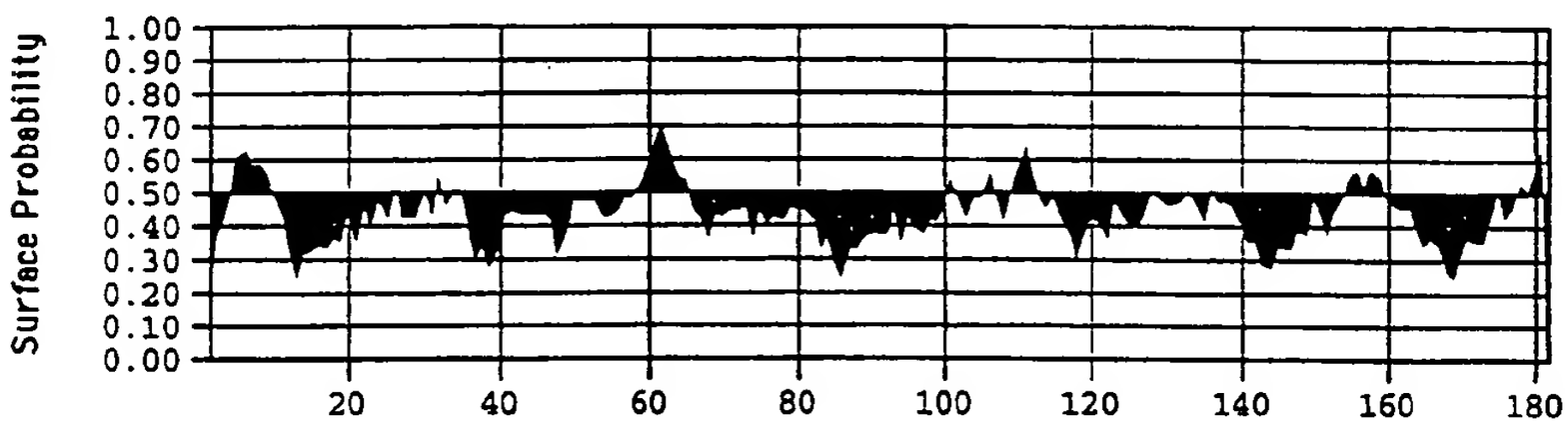


Fig. 7C

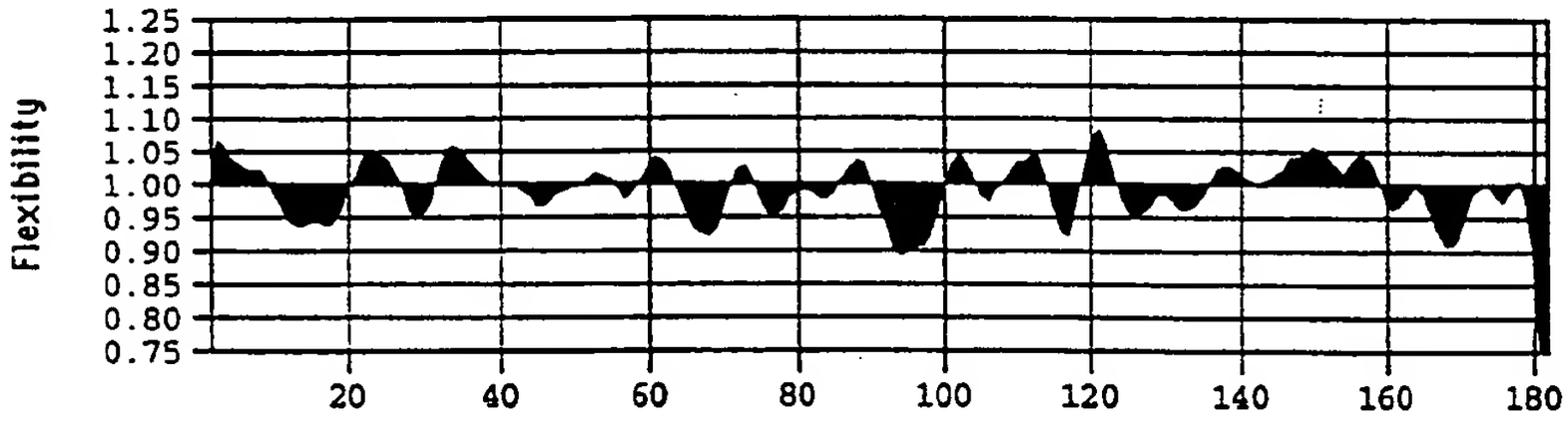


Fig. 7D

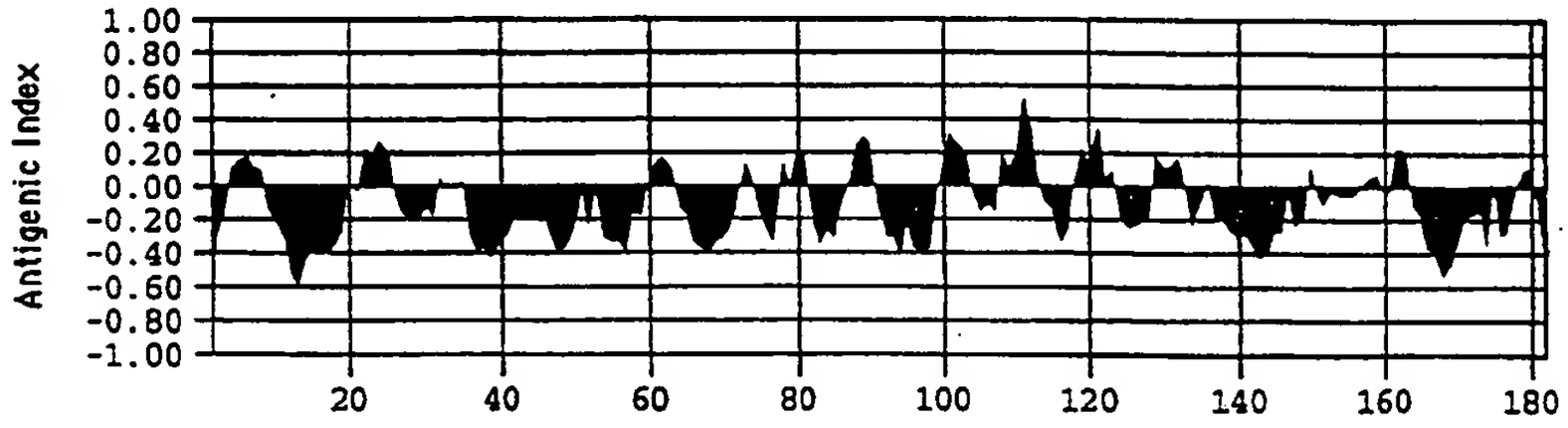


Fig. 8A

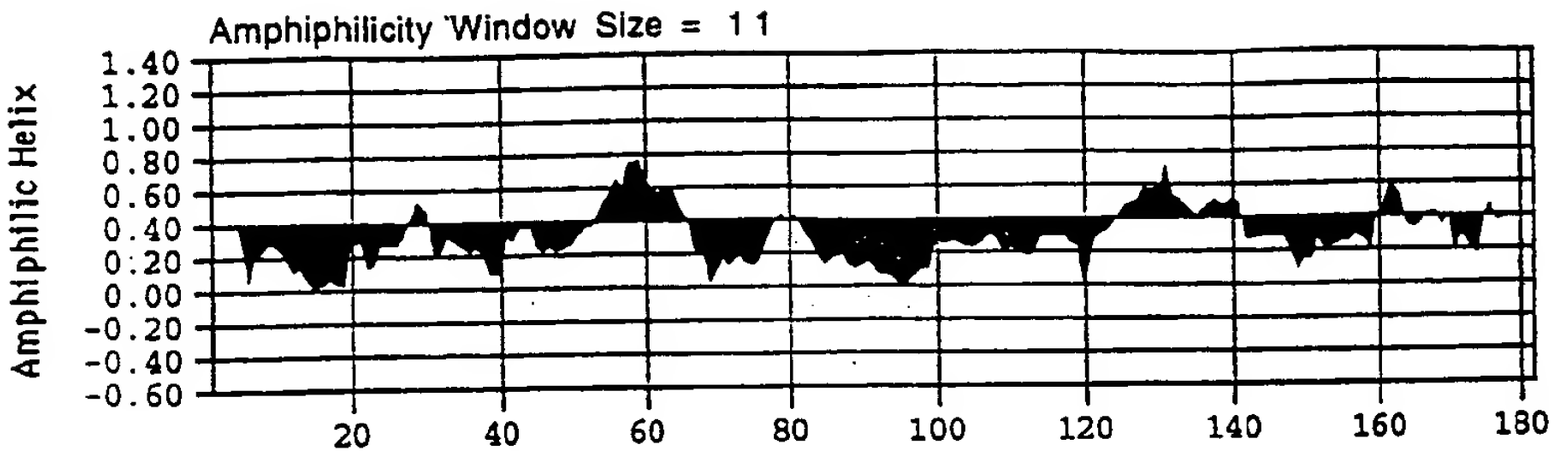


Fig. 8B

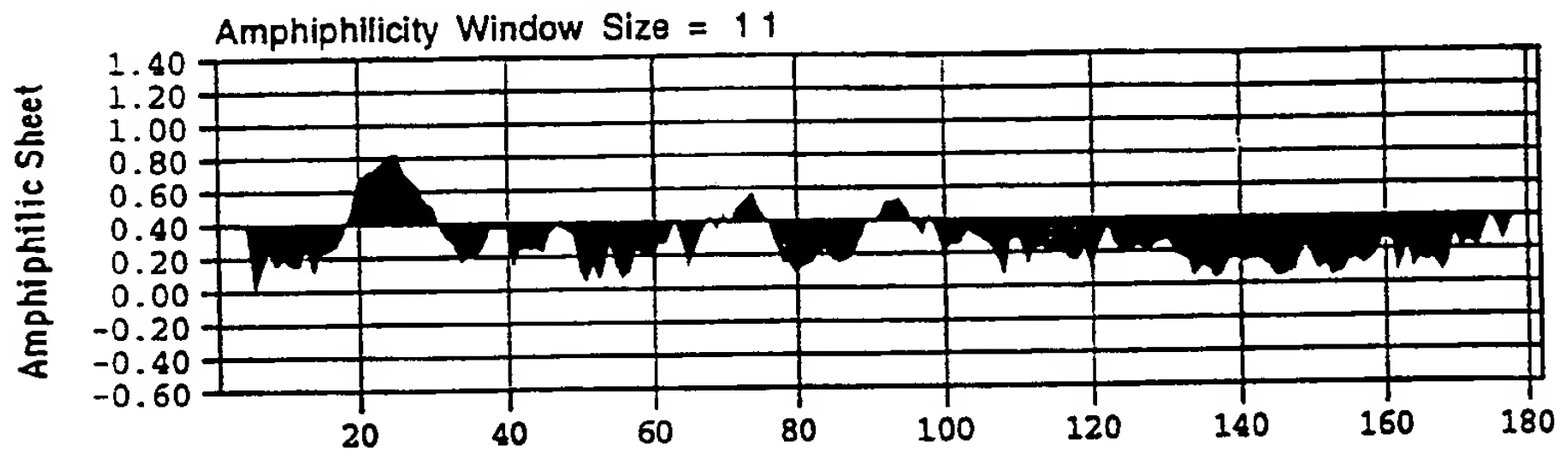


Fig. 8C

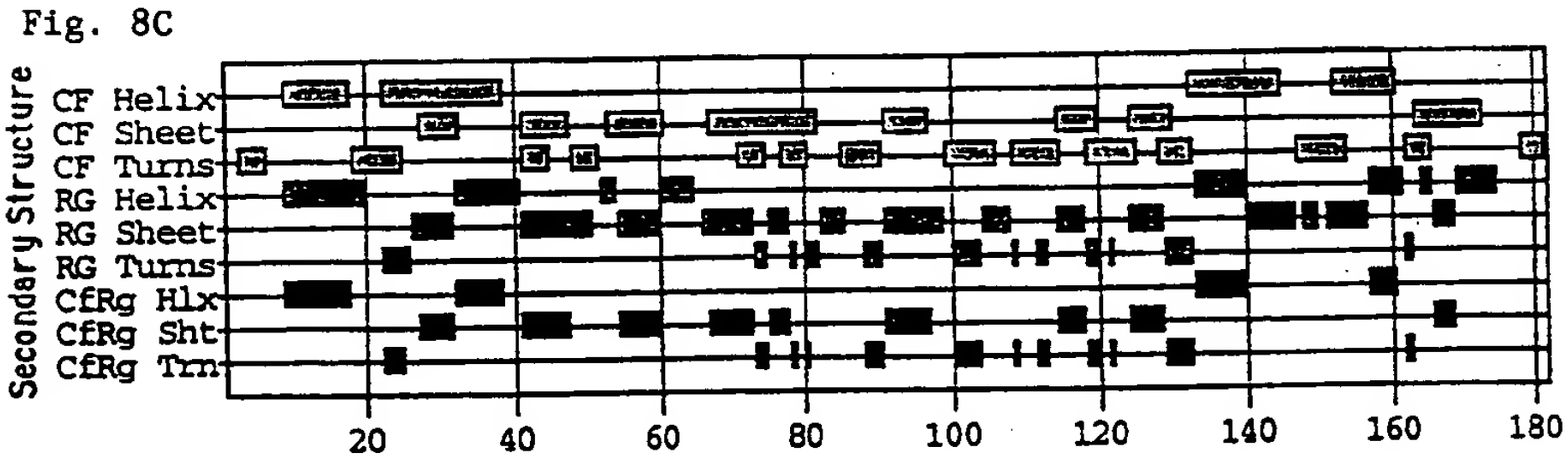


Fig. 9

```
Apomigren  MENDO  HENDO
IYSFDGRDIMTDPSWPQKVIWHGSSPHGVRLYDNYCEAWRTADTAVTGLASPLSTGKILDQKAYSCANRLIVLCIENSFMTDARK
IFSFDGRDYLRHPAWPQKSVWHGSDPSGRRLMESYCETWRTETTGATGQASSLLSGRLLEQKAASCHNSYIVLCIENSFMTSFSK
IFSFDGKDVLRHPTWPQKSVWHGSDPNGRRLTESYCETWRTEAPSATGQASSLLGGRLLGQSAASCHHAYIVLCIENSFMTASK
```

Fig. 10

FIG.12

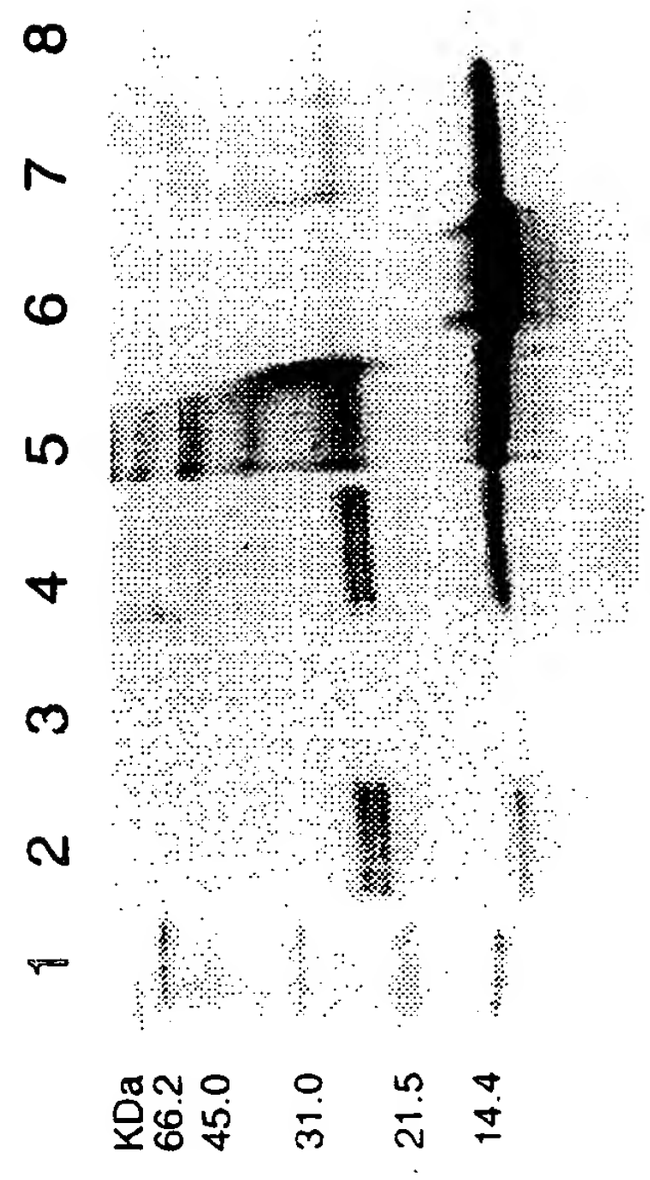

1 2 3 4 5 6 7 8
KDa
66.2
45.0
31.0
21.5
14.4

FIG.11

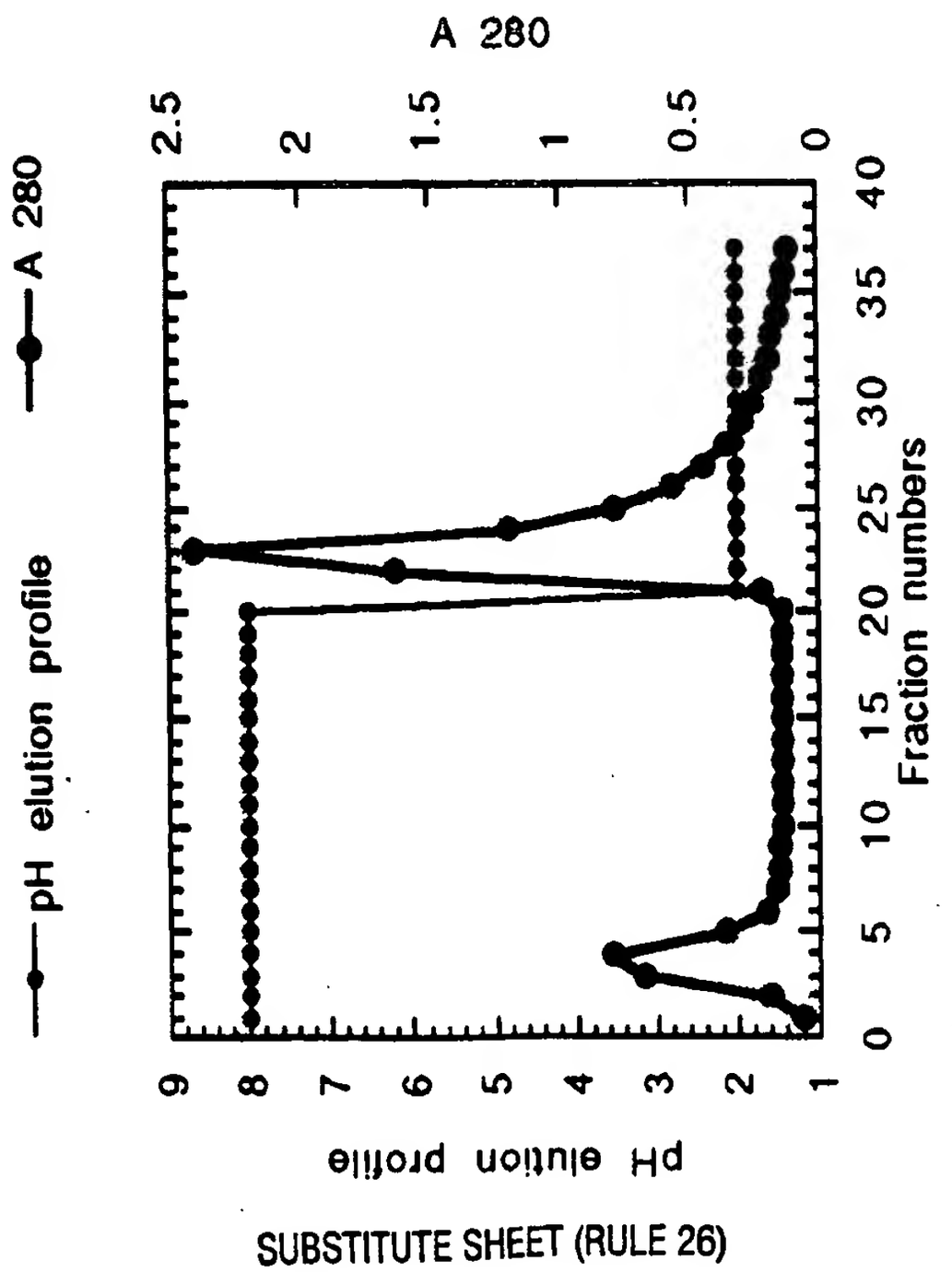
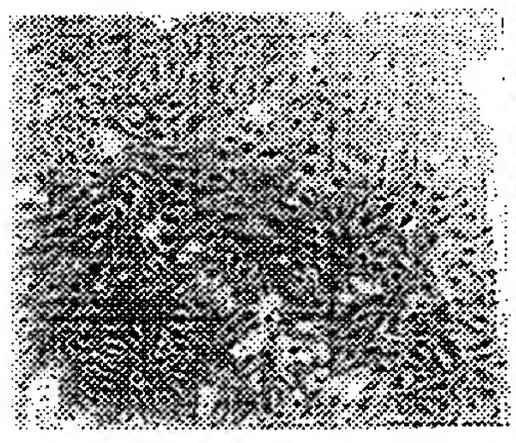
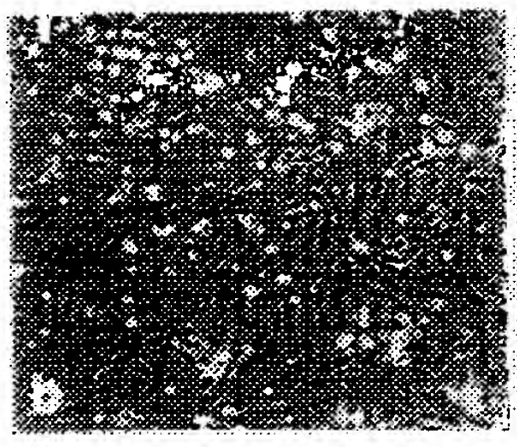

A 280
pH elution profile
A 280
2.5
2
1.5
1
0.5
0
0 5 10 15 20 25 30 35 40
Fraction numbers
9 8 7 6 5 4 3 2 1
pH elution profile

FIG.13A



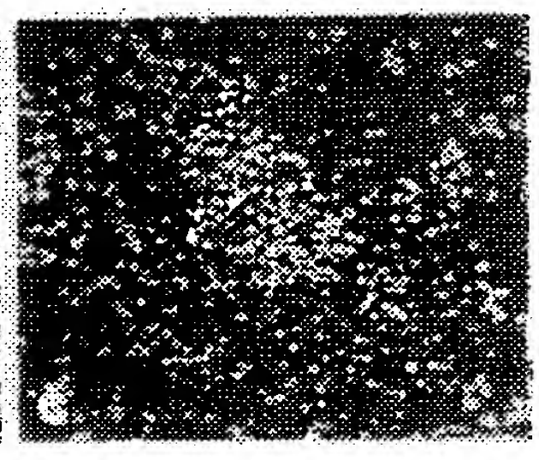
Control

FIG.13B



his.apomigren
0.05 µg/ml

FIG.13C



his.apomigren
0.7 µg/ml

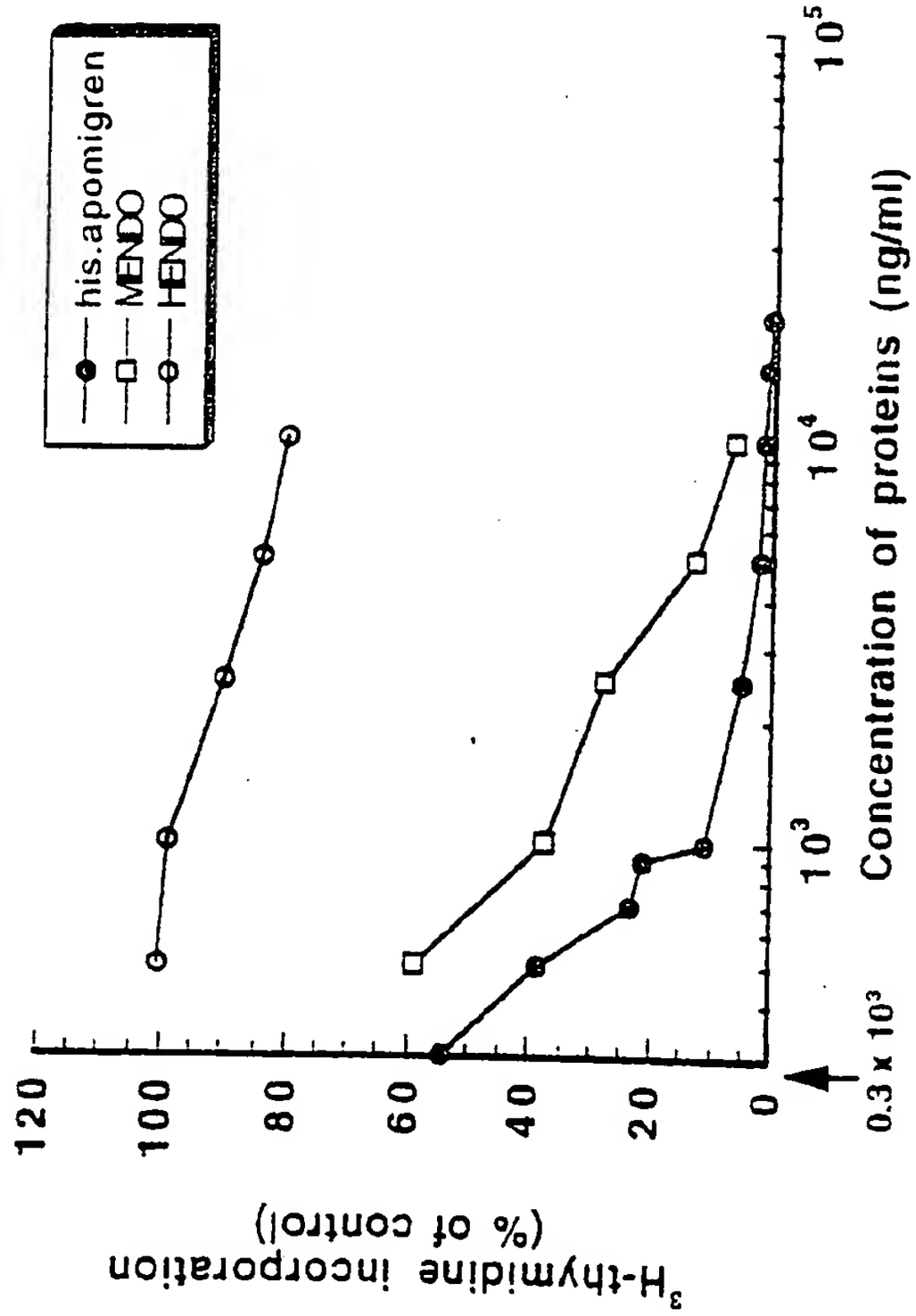

his.apomigren
MENDO
HENDO
120
100
80
60
40
20
0
0.3 x 10³
10³
10⁴
10⁵
Concentration of proteins (ng/ml)
³H-thymidine incorporation
(% of control)

Fig. 14

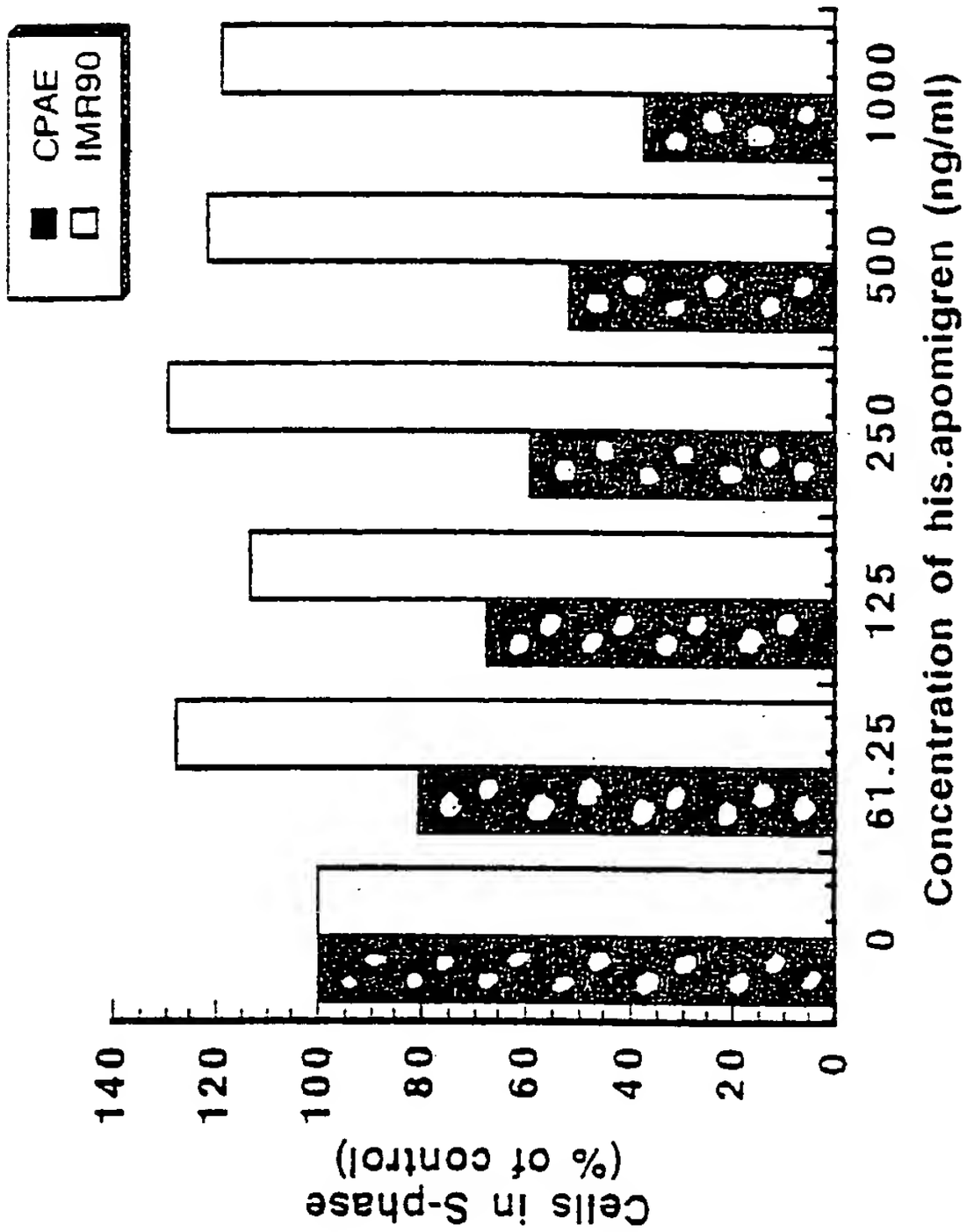

CPAE
IMR90
Cells in S-phase
(% of control)
140
120
100
80
60
40
20
0
0
61.25
125
250
500
1000
Concentration of his.apomigren (ng/ml)

Fig. 15

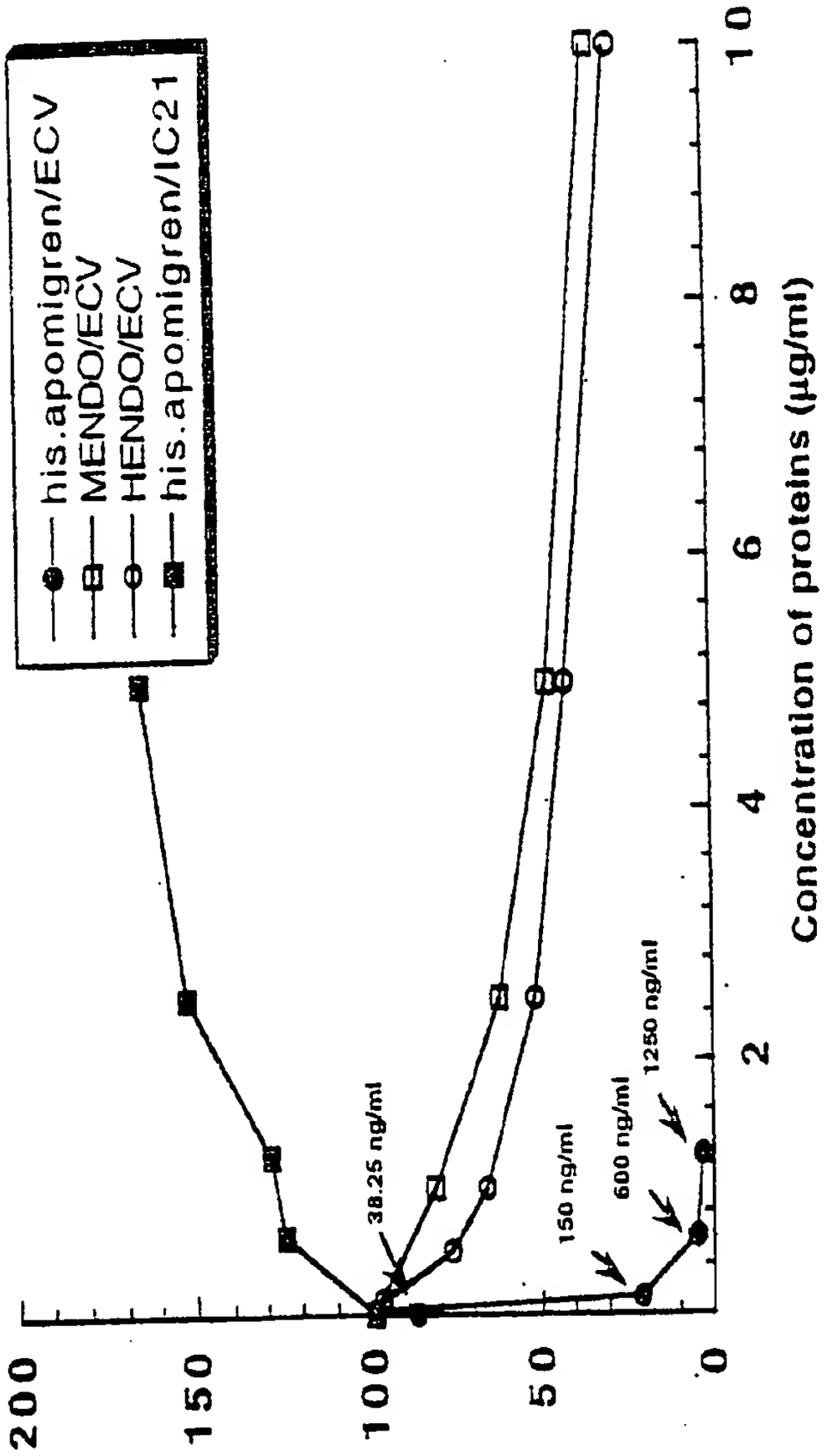

his.apomigren/ECV
MENDO/ECV
HENDO/ECV
his.apomigren/IC21
38.25 ng/ml
150 ng/ml
600 ng/ml
1250 ng/ml
0
2
4
6
8
10
Concentration of proteins (µg/ml)
0
5
10
15
20
No. of cells migrated
(% of control)

Fig. 16

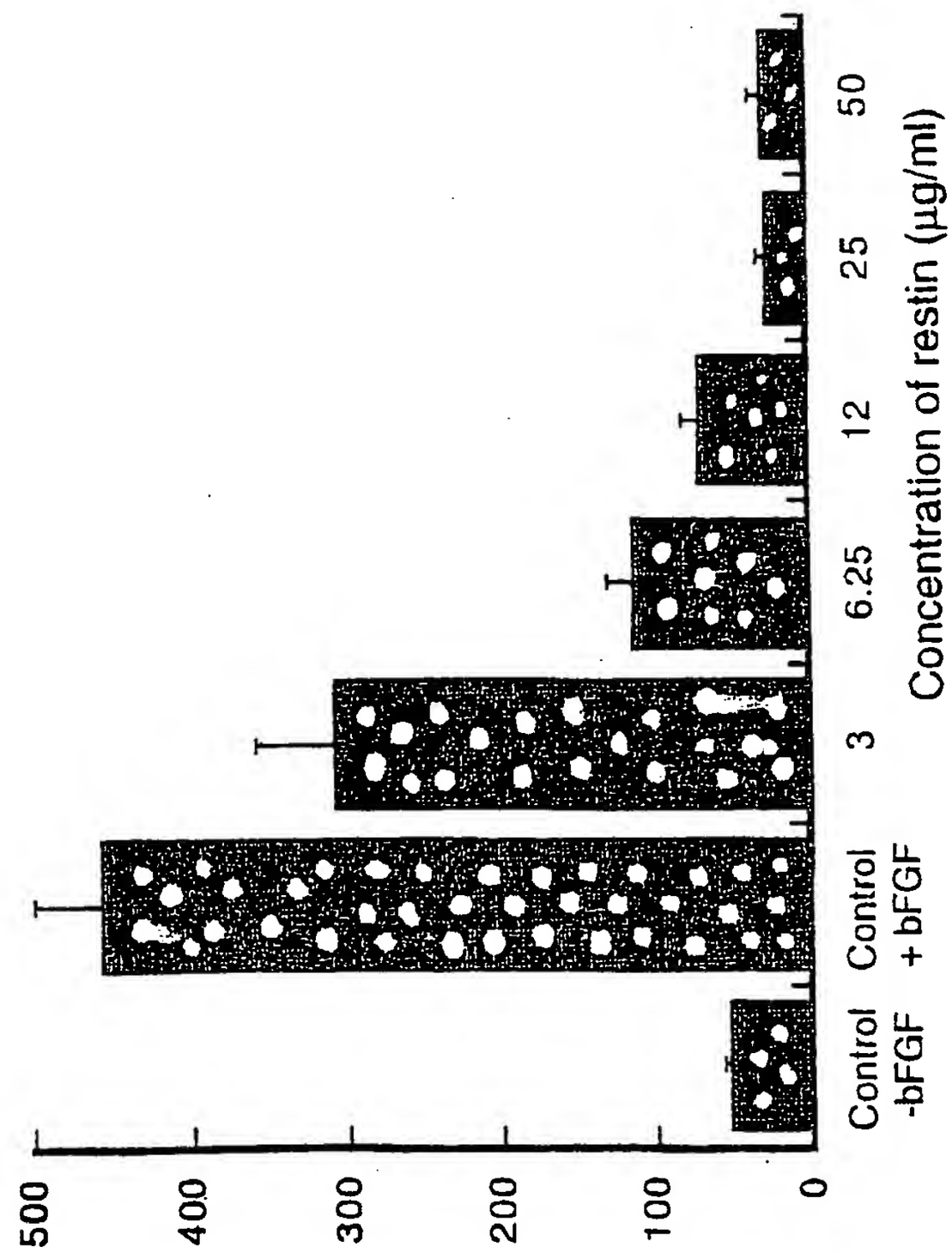

No. of cells migrated
0
100
200
300
400
500
Control -bFGF
Control + bFGF
3
6.25
12
25
50
Concentration of restin (µg/ml)

Fig. 17

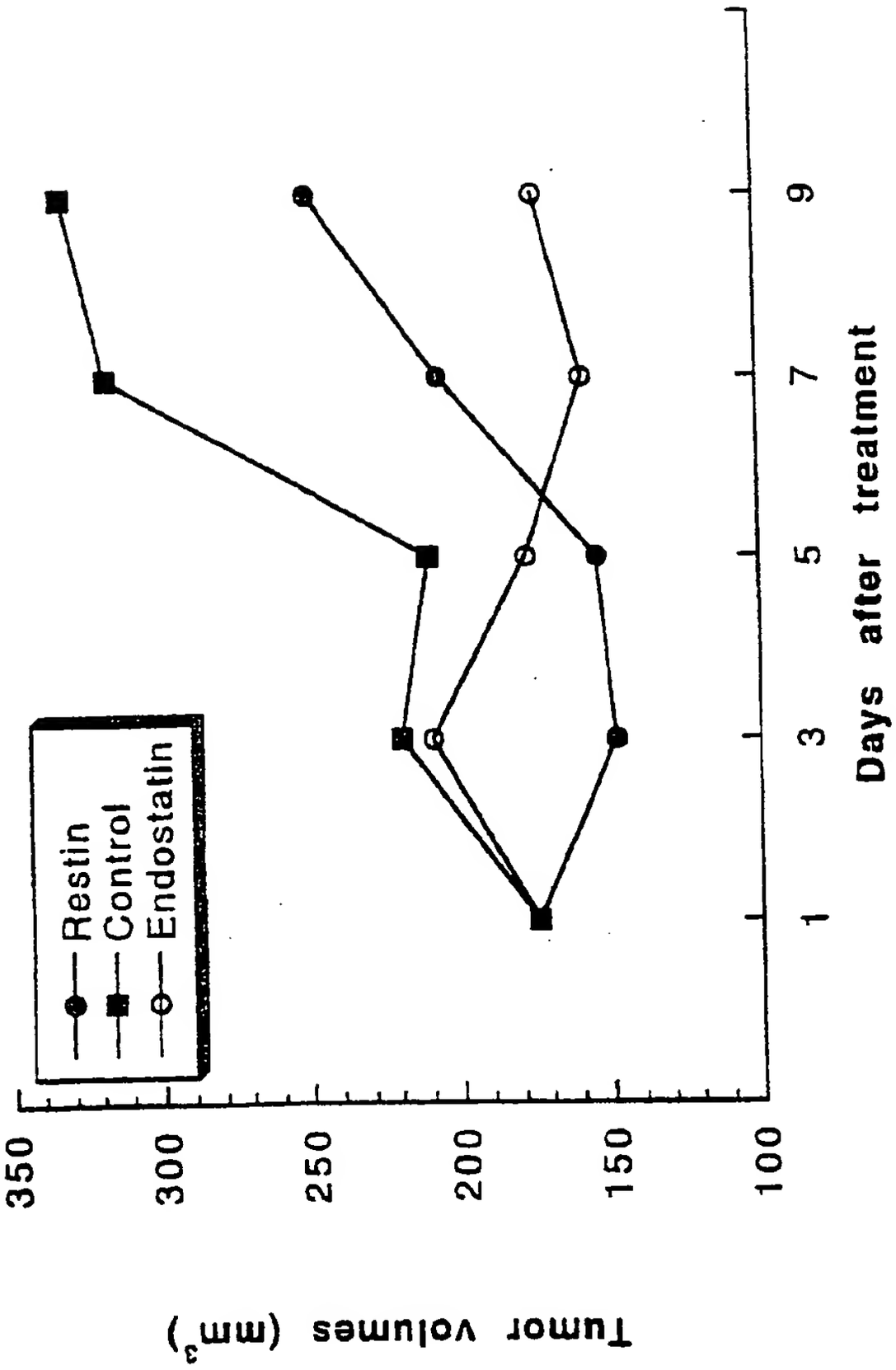

Restin
Control
Endostatin
350
300
250
200
150
100
1
3
5
7
9
Days after treatment
Tumor volumes (mm³)

Fig. 18

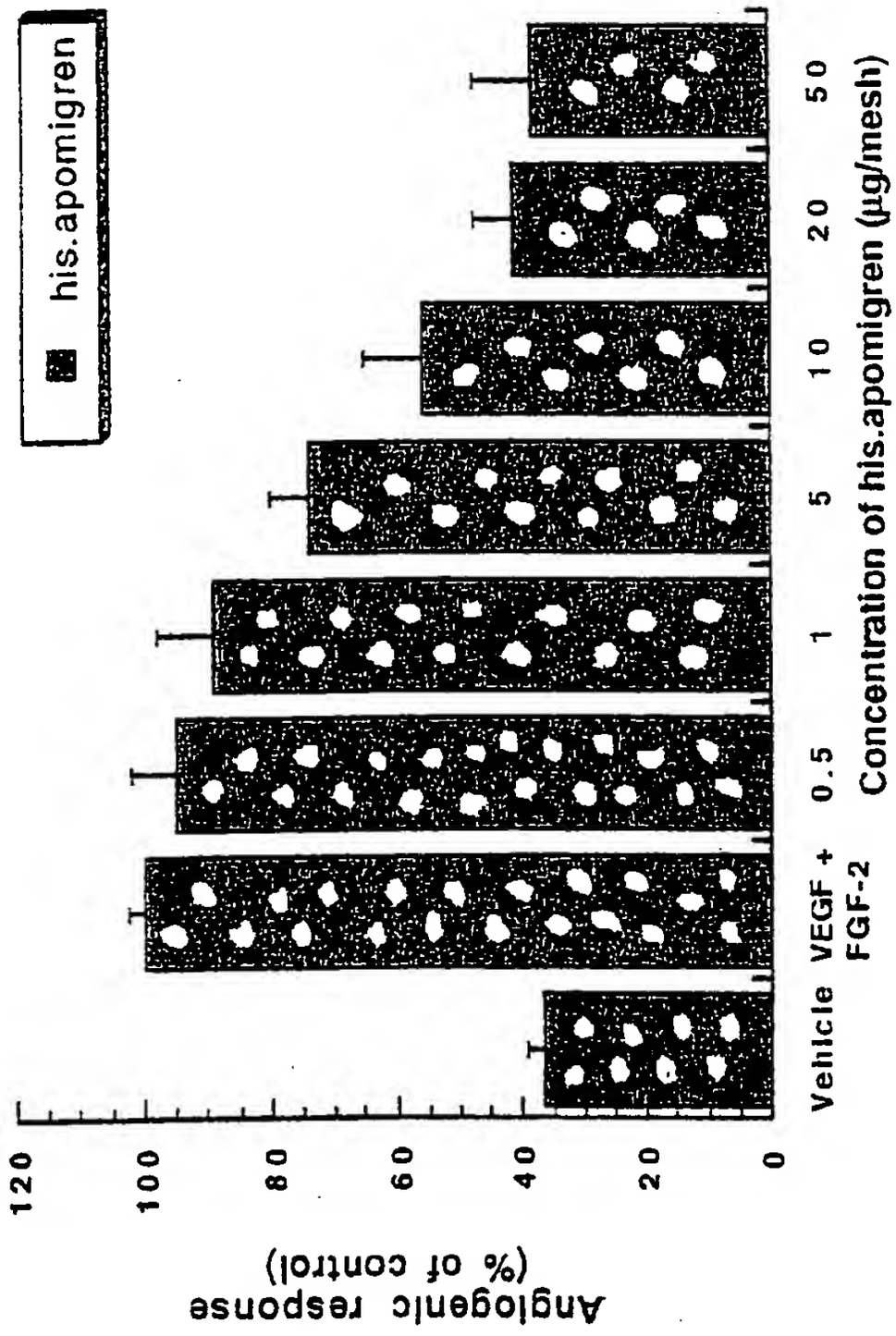

his.apomigren
Angiogenic response
(% of control)
0
20
40
60
80
100
120
Vehicle
VEGF + FGF-2
0.5
1
5
10
20
50
Concentration of his.apomigren (µg/mesh)

Fig. 19

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pET17b/ his.mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:7)	*Nde*I & *Xho*I	Prokaryotic expression, pET	MGHHHHHHHHHHSSGHIDDDDKH M-mendo (SEQ ID NO:9)
	5' AAC TCG AGC TAT TTG GAG AAA- GAG GT-3' (down) (SEQ ID NO:8)			
pET28a/ mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:7)	*Nde*I & *Not*I	Prokaryotic expression, pET	MGSSHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:11)
	5'-AAG CGG CCG CCT ATT TGG AGA-AAG AGG T-3' (down) (SEQ ID NO:10)			
pET28a/ EM-1	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:12)		Prokaryotic expression, pET	MGSSHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:11)
	5' TTA GCG GCC GCC TAC TCA ATG- CAC AGG ACG ATG TA-3' (down) (SEQ ID NO:13)			
pET28a/ EM-2	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:12)		Prokaryotic expression, pET	MGSSHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:11)
	5' TTA GCG GCC GCC TAG TTG TGG- CAG CTC GCA GCT TTC TG-3' (down) (SEQ ID NO:14)			

Fig. 20A

SUBSTITUTE SHEET (RULE 26)

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ mendo	5' GGG AAT TCC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:15)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA	EF-mendo
	5' AAG CGG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:10)			
pPICZαA/ His.mendo	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:16)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA	EFMGHHHHHHHHHHSSGHIDDDD KHM-mendo (SEQ ID NO:17)
	5' AAG CGG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:10)			
pPICZαA/ Hendo	5' TTT GAA TTC GCC CAC AGC CAC- CGC GAC TTC CAG CCG GTG CTC- CAC-3' (up) (SEQ ID NO:18)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA	EF-hendo
	5' AAA AGC GGC CGC CTA CTT GGA- GGC AGT CAT GAA GCT GTT CTC- AAT-3' (down) (SEQ ID NO:19)			
pPICZαA/ Restin	5' TTT TTT GAA TTC ATT TCA AGT- GCC AAT TAT GAG AAG CCT GCT- CTG CAT-TTG-3' (up) (SEQ ID NO:20)	*Eco*RI & *Not*I	Eukaryotic (Yeast), Pichia, pPICZαA	EF-restin
	5' AAG AAT GCG GCC GCT TAC TTC- CTA GCG TCT GTC ATG AAA CTG- TTT TCG AT-3' (down) (SEQ ID NO:21)			

Fig. 20B

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ His.Restin	5' AAT TCC ATC ACC ATC ACC ATC- ACG-3' (up) (SEQ ID NO:22) 5' AAT TCG TGA TGG TGA TGG TGA-TGG-3' (down) (SEQ ID NO:23)	*Eco*RI (oligo insertion)	Eukaryotic (Yeast), Pichia, pPICZαA	EFHHHHHH-restin (SEQ ID NO:24)
pET28a/ apomigren	5' TTT CAT ATG ATA TAC TCC TTT- GAT GGT CGA GAC ATA ATG ACA-3' (up) (SEQ ID NO:25) 5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)	*Nde*I & *Not*I	Prokaryotic, pET system	MGSSHHHHHHSSGLVPRGSHM-apomigren (SEQ ID NO:11)
pPICZαA/ apomigren	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:16) 5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)	*Eco*RI & *Not*I	Eukaryotic (Yeast), Pichia, pPICZαA	EFMGSSHHHHHHSSGLVPRGSHM-apomigren (SEQ ID NO:27)

Fig. 20C

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 98/26058

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/62 C12N5/10 C12N1/19 C07K14/78
C07K16/18 C12Q1/68 A61K48/00 A61K38/17 //(C12N1/19,
C12R1:84)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 15666 A (CHILDRENS MEDICAL CENTER) 1 May 1997 see abstract see page 1 - page 7 see page 13, line 7 - line 13 see page 14, line 23 - page 15, line 6 see examples 1-10 see claims 1-33 --- -/--	1,5,11, 13-17, 78-81, 86,87

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 April 1999	03/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 98/26058

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	REHN M. ET AL.: "Primary structure if the alphal chain of mouse type XVIII collagen, partial structure of the corresponding gene, and comparison of the alphal(XVIII) chain with its homologue, the alphal(XV) collagen chain" J. BIOL. CHEM., vol. 269, no. 19, 13 May 1994, pages 13929-13935, XP002099772 see the whole document	1-19,21, 23-87
A	---	20,22
Y	REILLY M.S. ET AL.: "Endostatin: an endogenous inhibitor of angiogenesis and tumor growth" CELL, vol. 88, 24 January 1997, pages 277-285, XP002099773 cited in the application see page 282, column 1	1-19,21, 23-87
P,X	--- RAMCHANDRAN R. ET AL.: "Antiangiogenic activity of Restin, NC10 domain of human collagen XV: comparison to endostatin" BIOCHEM. BIOPHYS. RES. COMM., vol. 255, 1999, pages 735-739, XP002099774 see the whole document	1-87
P,X	--- BACHELOT ET AL: "Retrovirus-mediated gene transfer of an angiostatin-endostatin fusion protein with enahnced anti-tumor properties in vivo" PROCEEDINGS OF THE 89TH. ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, NEW ORLEANS, LA, MARCH 28 - APRIL 1, 1998, no. VOL. 39, March 1998, page 271 XP002089298 AMERICAN ASSOCIATION FOR CANCER RESEARCH see the whole document	17,18
E	--- WO 99 16889 A (G.D. SEARLE) 8 Apr11 1999 see abstract seq. ID 76	1,11, 13-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/26058

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: claims 34-74 and, if a procedure in vivo is envisaged, claims 32-33 relate to methods of treatment of the body. The search is limited to the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ...nal Application No
PCT/US 98/26058

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9715666 A	01-05-1997	US 5854205 A	29-12-1998
		AU 7466696 A	15-05-1997
		CN 1202932 A	23-12-1998
		CZ 9801177 A	12-08-1998
		EP 0857210 A	12-08-1998
		NO 981803 A	17-06-1998
		NZ 321356 A	25-11-1998
WO 9916889 A	08-04-1999	NONE	